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## INTRODUCTION

Shock is a leading cause of death among American soldiers wounded in battle. For injuries which are not immediately lethal, most deaths result from hemorrhagic shock or from its late sequelae, septic shock and multiple organ failure. The critical time in shock appears to be the period during which the patient is being resuscitated. Resuscitation is associated with a massive activation of the inflammatory reaction, producing immunosuppression, and rendering the patient vulnerable to sepsis and its sequelae. The **goal** of this research program is to develop new treatments for hemorrhagic shock which can be administered before or during initial resuscitation. These treatments are intended to be applied by front-line responders on the battlefield (and first responders in civilian life) as well as by fixed facilities, such as Forward Surgical Teams or Field Hospitals. Such agents must be non-toxic and have a very broad therapeutic ratio, so that they can be given safely to injured soldiers, and must be easy to administer under combat conditions. In previous work, the xanthine oxidase inhibitor allopurinol was found to improve cardiac output and survival in a shock model (1). Also in earlier work, changes in cellular energy stores were defined. (2, 3, 4) In current work, glutamine and alanine-glutamine dipeptide have shown to enhance ATP recovery and suppress cytokine responses. (5, 6, 7) Studies continue on other agents, specifically as DHEA, crocetin, and insulin. (3, 4, 8, 9), omega-3 fatty acids, and arginine.

## BODY

It should be noted that progress towards the four research objectives, while significant, has been slower than desired. This is due to three factors. First, the microarray studies in the animal models have been slowed by the relocation of the laboratory of the collaborating investigators to new quarters in a newly-built research building. Second, authorization to proceed with human studies was not obtained until June, 2007. Third, there have been personnel resignations, and one turnover is ongoing (Dr. R. Yang, a key individual). On the other hand, the expenditures on the grant have been considerably behind projections. For that reason, we intend to request a no-cost extension of the grant from May, 2008, to May, 2009, to enable us to complete this work.

**Research objective #1:** Establish and validate the micro-array studies, using the animal model. *Deliverable:* A panel of genes that are reproducibly altered in white blood cells and in liver and muscle by shock and resuscitation.

1. To facilitate this work, further work was done to refine the parameters used in the whole-animal model of hemorrhagic shock. Preliminary studies relating to this work have been completed and submitted for publication. These relate to the animal model used, and to previously-employed agents. (See list of publications, below).
2. Collaboration was established with the laboratory of Dr. Peter Smith, Kansas University Medical Center, Kansas City, Kansas. This facility carries out micro-

- array analysis of both animal and human genomes. A number of preliminary studies have established and validated the method of sample preparation for these studies.
3. Microarray studies were carried out to define the group of between 15 and 30 signature genes significantly and reproducibly activated or suppressed in shock and resuscitation. Using a whole animal model of hemorrhagic shock, animals were divided into four groups: unshocked controls, shock without resuscitation, shock with resuscitation, and shock with resuscitation and arginine. The animals were subjected to shock by withdrawing blood over 10 minutes, to 20-25 mm Hg, and kept at that level for 30 minutes by periodic further withdrawal of blood. Resuscitation was with 28 ml/kg Ringer's lactate, given over 30 minutes, without any return of shed blood. Blood was drawn before shock, and at 1 hour and 4 hours following resuscitation. The microarray analysis was carried out in the following manner. White blood cells were isolated, RNA extracted, and the samples prepared for microarray analysis in Dr. Smith's laboratory. The microarray analysis has been completed, and data analysis is being currently carried out. Final results are not yet available.
  4. Preliminary studies were done using the SuperArray technology, a more limited but faster technique than full microarray analysis. These studies were originally focused on the effect of crocetin on the transcription factor NF- $\kappa$ B, which corresponds to decreased expression of the genes responsible for inflammation. The SuperArray technology tested the expression of genes related to the NF- $\kappa$ B signaling pathway. The arrays were in 96-well blocks with primers pre-coated on the surface of the wells. There were 84 genes plus five housekeeping genes and two negative control genes. The same animal model was employed as described above, except for the sampling times. Blood samples were taken at baseline and 30 minutes after resuscitation. White cells were isolated for RNA extraction. Samples were processed using two-step real-time PCR technology, according to the manufacturer's recommendations (SuperArray Bioscience Corporation, Frederick, MD). Samples were run in the same system (DNA engine Opticon 2 real-time PCR detection system, Bio-Rad Laboratories, Inc., Hercules, Calif), and the cycles were determined at the same threshold number. The relative expression was analyzed with  $\Delta\Delta$ Ct method, and further processed in an Excel-based data analysis template. Sham (without shock) and shock (without resuscitation) animals have few/little genes up-down-regulation compared to animals resuscitated with LR after hemorrhagic shock. The genes up-and down-regulated fold are shown graphically in Figure 1 (for reperfusion with LR), with genes up-regulated more than 3-fold are listed in Table 1.
  5. To validate the microarray analysis, PCR studies are being carried out for individual cytokine genes, a technology which is well-established in our laboratory. Other measures of cellular response and cellular damage are being assessed, including in the animal model used, correlating these responses with the response shown by the signature genes. Analysis of I $\kappa$ B and nF $\kappa$ B using Western blot is being carried out. Finally, direct assay of cytokines is being done.
  6. A new model has been developed to further assess the cellular response to ischemia and re-oxygenation, a response which is at the heart of the response to

hemorrhagic shock. This model is based on exposing cultured macrophages to ischemia in a sealed chamber, and then re-exposing them to levels of oxygen found in room air (21%) For these experiments, it has been determined that reducing the level of oxygen to 1.5% from the usual 21% produces a significant hypoxic insult, but without killing the cells.. This corresponds to a partial pressure of oxygen of 10 mm Hg. Examination of this model is in the preliminary stages, but several significant observations have been made.

- a. First, cells exposed to this for 24 hours show less growth than control cells, and then recover and grow at a rate comparable with controls after being exposed to a normal oxygen level (21%, or about 140 mm Hg partial pressure).
- b. Second, measuring energy levels within the cells shows that ATP in the ischemic cells is depressed at the end of the ischemic period. After re-oxygenation, the ischemic cells recover ATP, when compared with cells kept ischemic for the entire experiment. Significantly, such cells show ATP levels which are still less than cells which were kept at 21% oxygen and not exposed to hypoxia. There is a similar finding in whole animal shock, following which ATP recovery is slow and incomplete compared with un-shocked controls.
- c. Third, stimulating the cells (with lipo-polysaccharide) during the re-oxygenation process produces elevated levels of caspase 3 and 7 compared with both normal oxygen controls and with hypoxic controls. This is consistent with increased caspase activation and increased apoptosis seen in the whole animal shock model. It suggests that it is re-oxygenation rather than ischemia which is the stimulus to apoptosis.
- d. Studies of cytokine response to LPS are ongoing, and will include TNF-alpha and IL-2 beta.
- e. Further examination of this model is planned. It will allow characterization of proposed therapeutic agents in terms of ATP recovery, caspase activation, and cytokine response. It is expected that gene array analysis of this model will allow a much more precise characterization of the response of the macrophage genome, as opposed to the response which will be measured .

Figure 1. Genes related to NF- $\kappa$ B, following shock and resuscitation, showing both up- and down- regulation as indicated by the direction of the bars. Shock plus resuscitation with LR and arginine is compared with an u-nshocked control.

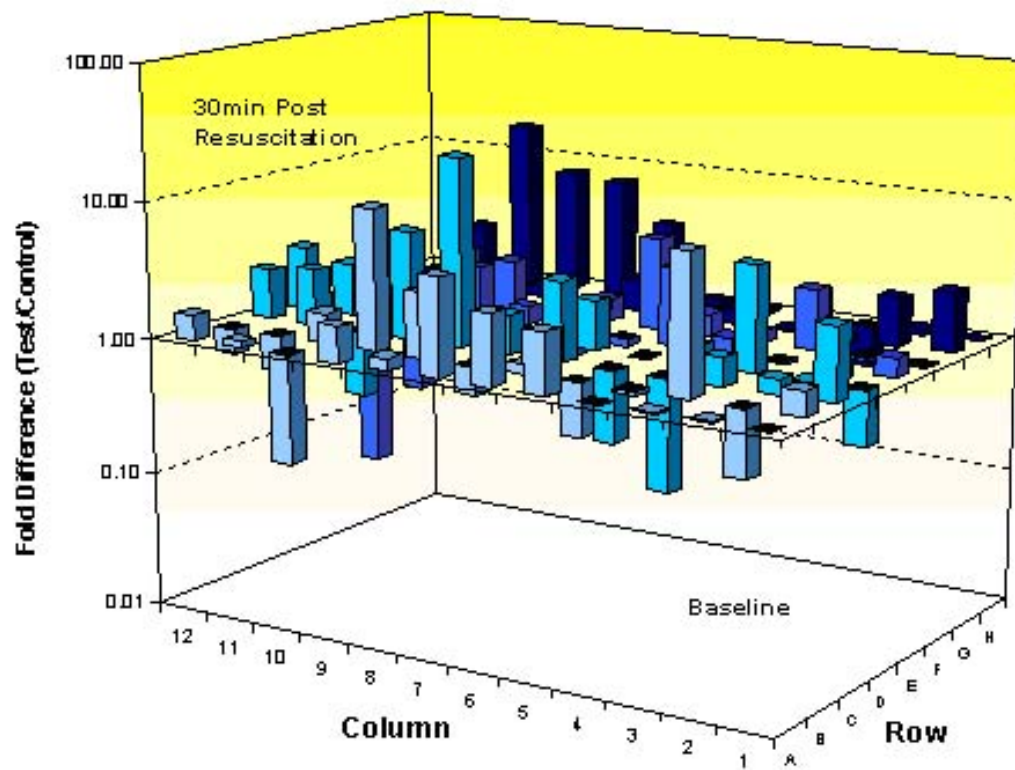


Table I. Genes up- or down-regulated more than threefold, in the whole animal model of hemorrhagic shock in the rat, compared with un-shocked control.

NF- $\kappa$ B Signaling Pathway RT <sup>2</sup> Profiler PCR Arrays	
Description	Fold Up- or Down-Regulation
	30min Post Resuscitation /Baseline
BCL2-antagonist of cell death	3.26
BCL2-associated X protein	5.20
Breast cancer 1, early onset	-6.30
Cyclin-dependent kinase 2	-3.03
Cyclin-dependent kinase 4	10.27
E2F transcription factor 1	10.62
Fibroblast growth factor receptor 2	3.39
HIV-1 Tat interactive protein 2, 30kDa	-6.51
Interferon, alpha 1	-3.33
Interferon, beta 1, fibroblast	3.78
Interleukin 8	23.01
Integrin, alpha 1	6.18
Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	5.86
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	3.71
Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 5	4.58
Telomerase reverse transcriptase	-5.53
Transforming growth factor, beta 1 (Camurati-Engelmann disease)	-23.85



**Research objective #2:** Define the effect on gene expression of the agents which will be used clinically, using the animal model. *Deliverable:* The effect of the agents upon the level of expression of the signature genes.

1. The genetic studies on the agents to be used clinically are in progress. Once the results from the shock experiments are identified, then all four agents will be used in the animal model, to determine their effects on the genome.
2. The choice of the agents has been refined. Crocetin is still in early Phase I studies, and will be unavailable for human studies within the timeline of this study. Alanine-glutamine and glutamine have been investigated in the whole animal model of shock. While both have produced better short-term survival than controls in the whole-animal model of shock, glutamine has been shown to be more effective. Arginine has been investigated, and also produces enhanced short-term survival compared with controls in the whole-animal model of shock. Three other agents have been considered. Allopurinol was shown to provide survival benefit in the canine shock model 20 years ago. (1) This agent has been used to ameliorate the harmful effects of hypoxia in harvested organs to be transplanted (as in the standard UW Solution) and in cold cardioplegia during aortic valve surgery. It appears to act by inhibiting xanthine oxidase, and hence lowering the production of free radicals during reperfusion. It is now available in an intravenous form approved for human use, a form which is very similar to that used in the earlier studies. It will be used in the whole animal model, and will probably be recommended for human studies. Mevinolin (Lovastatin), which has been used in experimental septic shock by Dr. Qureshi, is a strong candidate for this study, as it appears to moderate the cytokine response to lipo-polysaccharide in the mouse model, probably through a proteasomal mechanism (12). However, its effect on the whole-animal model of shock has not been determined. Its use in this model or in patients is questionable, since it is not available in an intravenous preparation. Dihydro-epi-andosterone (DHEA) was used in a whole-animal porcine model, but its use was disappointing, and its further use in patients, was not recommended on the basis of this work. (9)
3. Studies of omega-3 fatty acids were begun as part of a different project, but were analyzed and are briefly presented here. Briefly, rats were fed for four weeks with a rat chow diet high in omega-3 fatty acids, then subjected to a whole animal model of hemorrhagic shock as described above. Controls were fed usual rat chow. Sham controls were also studied. There was no survival advantage in the omega-3 fed animals. However, differences were seen in the amount of lung edema, and in gene expression for interleukin 1-beta and heat shock protein-25, both measured by mRNA levels using real time polymerase chain reaction. (Figures 2, 3, 4) No differences were seen in tumor necrosis factor alpha, or in inducible nitric oxide synthase. The results were consistent with the hypothesis that feeding with omega-3 fatty acids minimizes the inflammatory response to hemorrhagic shock and resuscitation. However, because there was no survival advantage even with prolonged pre-shock feeding, it was felt that further studies directed towards to use of omega-3 fatty acids during resuscitation would not be productive. The agent remains of significant interest, but not in this context.

4. Survival studies with glutamine, arginine-glutamine dipeptide, and arginine has been carried out over the past year. The primary limitation of survival studies in the rat model is that the primary endpoint is short term survival, at 2 and 24 hours. The rat does not exhibit late post-resuscitation organ failure as may be seen in patients. Having said this, these studies are nonetheless important because they provide a basis for the human therapeutic use of these agents.
5. Glutamine studies are presented in this table. (See also Figure 2)

<b>Survival with or without Glutamine in Resuscitation</b>		
	<b>LR</b>	<b>Glutamine</b>
<b>Rat weight (gram)</b>	295.83±3.03	293.00±3.84
<b>Cannulating Time (minutes)</b>	6.50±0.29	6.50±0.23
<b>Bleeding (ml)</b>	9.22±0.41	9.05±0.58
<b>*Survival &gt; 24 hours</b>	4/12	11/12

Shock at MAP = 22±4 mmHg, 90 minutes. Resuscitated with Ringer's Lactate (LR, 21ml/kg) with/without glutamine (630mg/kg) over 30 minutes. Data expressed as mean ± SEM. \*P< 0.05 (LR vs Glutamine).

6. Alanine-glutamine dipeptide (AGD) studies are presented here. (See also Figure 3) It should be noted that AGD is used widely in the clinical situation (in Europe, not in the US), and that it appears to be cleaved to alanine and glutamine once it enters the circulation. It is thus considered to be therapeutically equivalent to glutamine. However, the present studies show that, while AGD is definitely of benefit, it does not have as pronounced an effect on survival as glutamine.

<b>Survival with or without AGD in Resuscitation</b>		
	<b>LR</b>	<b>AGD</b>
<b>Rat weight (gram)</b>	294.61±2.47	297.58±3.48
<b>Cannulating Time (minutes)</b>	6.78±0.27	6.58±0.29
<b>Bleeding (ml)</b>	10.14±0.29	10.93±0.43
<b>*Survival &lt;24 hours</b>	10/18	4/18
<b>Survival &gt; 24 hours</b>	6/18	9/18

Shock at MAP = 25±4 mmHg, 90 minutes. Resuscitated with Ringer's Lactate (LR, 2ml) with/without Alanine-glutamine dipeptide (AGD 936mg/kg, equivalent with glutamine 630mg/kg) over 10 minutes. Data expressed as mean ± SEM. \*P< 0.05 (LR vs AGD).

7. Arginine studies are presented here. (See also Figure 4) In this model, arginine was nearly as effective as glutamine.

<b>Survival with or without Arginine in Resuscitation</b>		
	<b>LR</b>	<b>Arginine</b>
<b>Rat weight (gram)</b>	286.70±4.03	294.40±4.81
<b>Cannulating Time (minutes)</b>	6.35±0.13	6.80±0.17
<b>Bleeding (ml)</b>	9.32±0.31	10.06±0.35
<b>*Survival &lt; 24 hours</b>	11/20	4/20
<b>Survival &gt; 72 hours</b>	6/20	12/20

Shock at MAP = 20±4 mmHg, 90 minutes. Resuscitated with Ringer's Lactate (LR, 21ml/kg) with/without arginine (300mg/kg) over 30 minutes. Data expressed as mean ± SEM. \*P< 0.05 (LR vs Arginine).

8. Figures 2, 3, and 4 show graphically the summary of the series of experiments. In these experiments, animals were bled to a pressure of 23 mm Hg over 10 minutes, then kept hypotensive by periodic withdrawal or reinfusion of blood for 90 minutes. They were then resuscitated with a small amount of Ringer's lactate (6 ml/kg) with or without the therapeutic agent, over a period of 15 minutes. The animals were awakened, and kept in cages with supplemental heating and adequate water. If they were able, they were fed the next morning. Figure 2 shows that survival at 24 hours was significantly better in the glutamine group. As can be seen, the pressures diverged significantly during the resuscitation phase. Data for the AGD and arginine studies are graphically shown in Figures 3 and 4.

Figure 2: Shock Survival Study, with Glutamine.

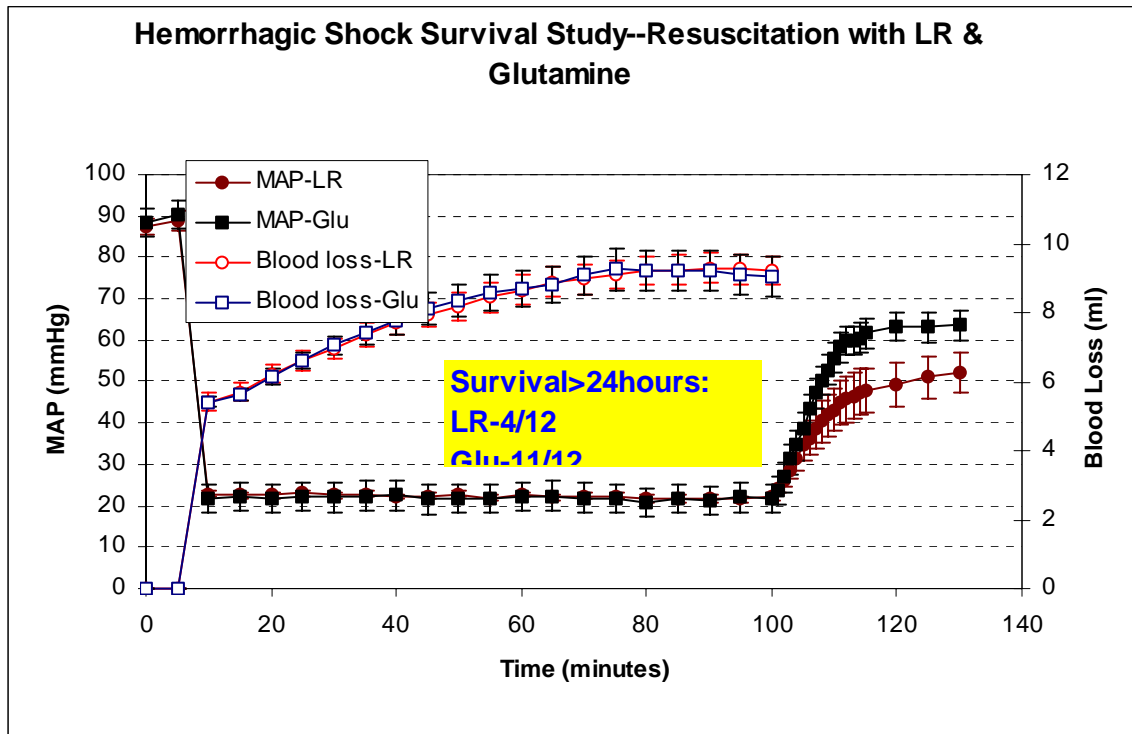


Figure 3: Shock survival study with alanine-glutamine dipeptide (AGD)

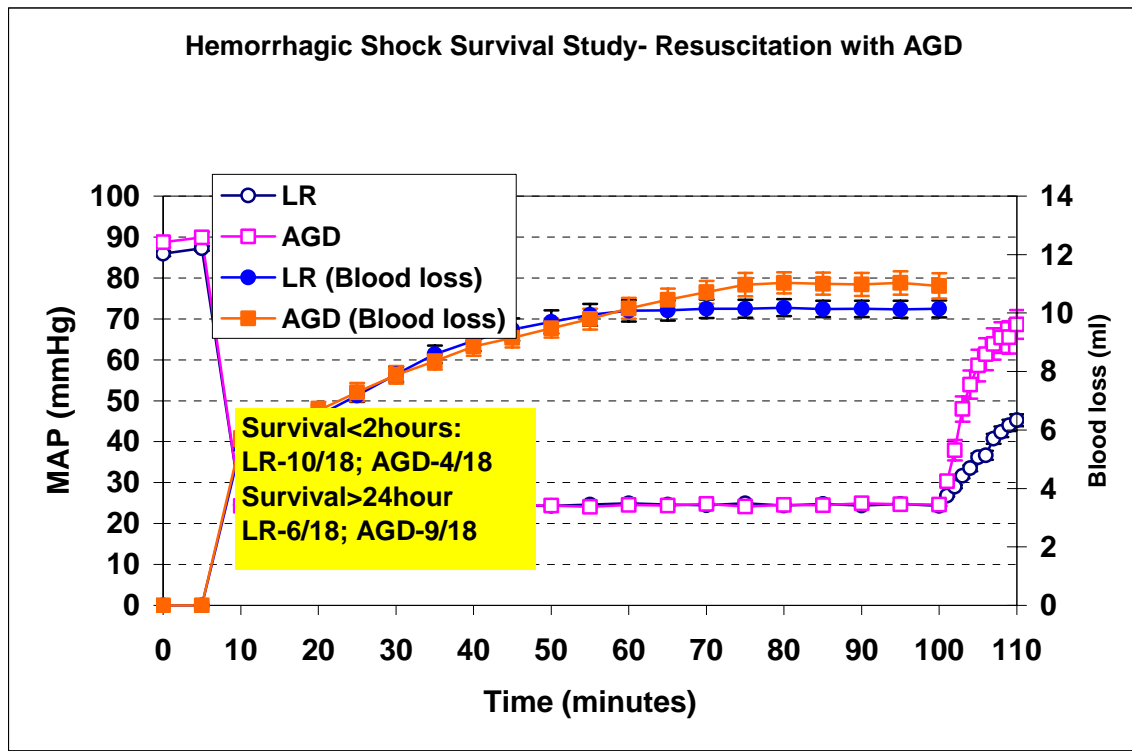
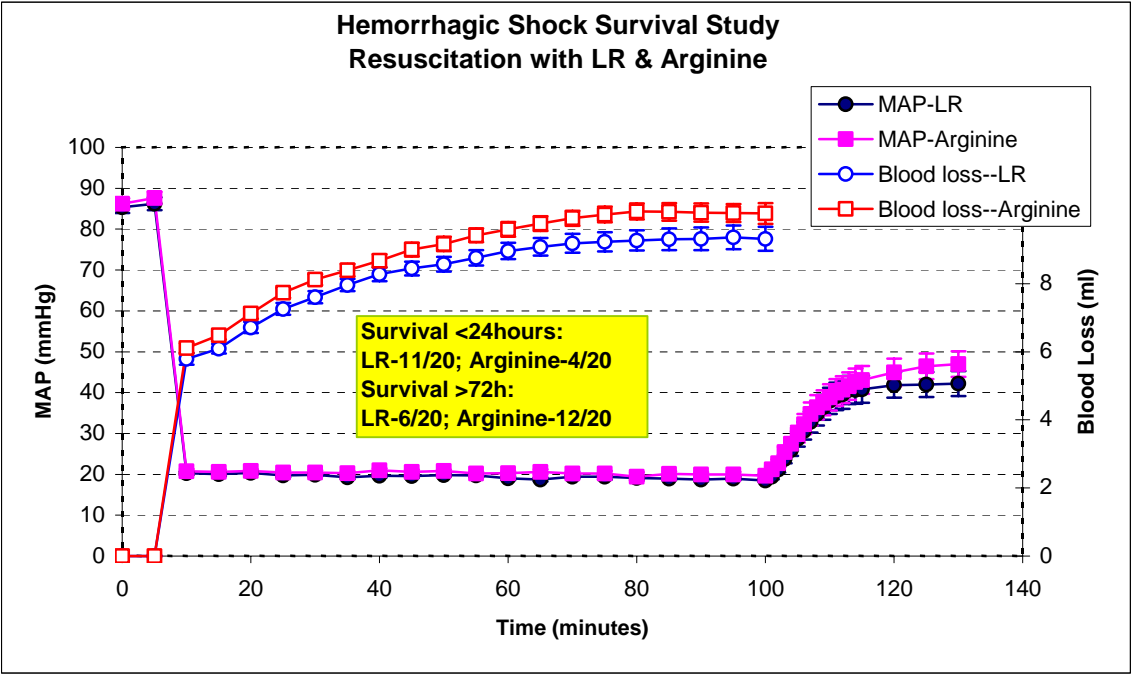


Figure 4: Shock survival study with arginine.



**Research objective #3:** Assess gene expression in patients treated with current initial resuscitation protocols. *Deliverable:* Coordinated with objective #1, A panel of genes that are reproducibly altered in white blood cells and in liver and muscle by shock and resuscitation.

1. The experimental protocol was approved by the Institutional Review Board of the University of Missouri – Kansas City. It has taken a considerable amount of time for the pre-approval review by the Human Research Protection Office (HRPO), Office of Research Protections (ORP), U.S. Army Medical Research and Materiel Command (USAMRMC). However, approval has just been obtained, as of June 7, 2007. Research on this will proceed.
2. Under this protocol, blood samples will be taken from patients arriving in the trauma center who exhibit blood loss or signs of hypovolemia. Blood samples will be collected one and four hours after resuscitation. Tissue samples (liver and muscle) will be taken if the patient is brought to the operating room for immediate operative therapy. These samples will then be processed and submitted for microarray analysis.

**Research objective #4:** Assess the response of patients to administration of modified resuscitation procedures. *Deliverable:* The effect of the experimental agents upon signature genes, cellular response, and cellular damage in patients.

This objective has not been started, as its initiation has to follow completion earlier objectives. It is proposed to begin the approval process, however, since it is anticipated that approval of this phase will consume approximately a year. It appears, on the basis of work carried out so far, that glutamine, arginine, and allopurinol will be the agents selected for human use. All three have shown improvement in survival in whole-animal models of shock, all can be given intravenously, and all are safe when given to humans.

## **KEY RESEARCH ACCOMPLISHMENTS**

- Further refinement of whole-animal model of hemorrhagic shock
- Initial development of ischemic cell-cultured macrophage model of hemorrhagic shock
- Survival studies using glutamine in the whole-animal model of hemorrhagic shock
- Survival studies using alanine-glutamine dipeptide in the whole-animal model of hemorrhagic shock
- Survival studies using arginine in the whole-animal model of hemorrhagic shock
- Gene micro-array studies of white blood cells in the whole-animal model of hemorrhagic shock
- Gene micro-array studies of the response to arginine in the whole-animal model of hemorrhagic shock

## REPORTABLE OUTCOMES

### PUBLICATIONS AND PRESENTATIONS

Yang R, Tan X, Thomas AM, Shen J, Qureshi N, Morrison DC, Van Way CW 3<sup>rd</sup>. Crocetin inhibits mRNA expression for tumor necrosis factor-alpha, interleukin-1beta, and inducible nitric oxide synthase in hemorrhagic shock. *JPEN J Parenter Enteral Nutr.* 2006 Jul-Aug;30(4):297-301. (Attached)

Yang R, Martin L, Woodall C, Thomas AM, Qureshi N, Morrison DC, Van Way CW III. Administration of glutamine following hemorrhagic shock restores cellular energy, reduces cell apoptosis and damage, and increases survival. *JPEN J Parenter Enteral Nutr.* 31:94-100,2007. (Attached)

Yang R, Tan X, Thomas AM, Qureshi N, Morrison DC, Van Way CW III.: Alanine-Glutamine Dipeptide (AGD) Inhibits Expression of Inflammation-Related Genes in Hemorrhagic Shock. *JPEN Jour Parent Ent Nutr* 31:32-36, 2007. (Attached)

Yang R, Tibbs BM, Chang B, Nguyen C, Woodall C, Steppacher R, Helling T, Van Way CW 3<sup>rd</sup>. Effect of DHEA on the hemodynamic response to resuscitation in a porcine model of hemorrhagic shock. *J Trauma.* 2006 Dec;61(6):1343-9. (Attached)

Yang R, Van Way CW III, Tan X, Thomas A, Shen J, Steppacher R, Qureshi N, Morrison DC. Alanine-glutamine dipeptide inhibits expression of inflammation-related genes in hemorrhagic shock. *Clinical Nutrition Week*, Dallas. February 12-15, 2006. (Oral presentation). *Clinical Nutrition week.* 2006, p145. (Abstract of Distinction)

Thomas A, Yang R, Van Way CW III, Quresh N. A Preliminary micro-array study of the response to hemorrhagic shock and to therapy with crocetin and alanine-glutamine dipeptide. *Kansas City Area Life Sciences.* March 30, 2006. *Kansas City Life Sciences.* 2006, p163. (Poster and Abstract)

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## CONCLUSIONS

To the present time, this project has resulted in the identification of three agents which will be suitable for administration to the injured patient during the initial resuscitation phase: glutamine, arginine, and allopurinol. Practical considerations appear to eliminate crocetin (unapproved drug), mevinolin (no intravenous form), DHEA (poor effect, toxic), and alanine-glutamine dipeptide (effective, but less so than glutamine). Further studies in the whole-animal model continue. A non-animal cell culture model has been developed, and will be further characterized. It appears to have great potential for screening possible agents to be given during resuscitation.

Studies of gene microarray analysis in the whole animal model have proceeded more slowly than expected, but the first set of experiments have been carried out, and the data is presently being analyzed to identify the subset of genes upon which further studies will focus. Gene studies in the human patient have been delayed by the requirements of the approval process, but this has recently been obtained (June 7, 2007), and these studies will proceed.

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8. Yang R, Tan X, Thomas AM, Shen J, Qureshi N, Morrison DC, Van Way CW III. Crocetin Inhibits mRNA Expression for Tumor Necrosis Factor- $\alpha$ , Interleukin-1 $\beta$  and Inducible Nitric Oxide Synthase in Hemorrhagic Shock. *Jour Parent Ent Nutr* 30:297-301, 2006.
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## APPENDICES

### MANUSCRIPTS PUBLISHED OR IN PRESS (36 PAGES TOTAL)

- I. Yang R, Tan X, Thomas AM, Shen J, Qureshi N, Morrison DC, Van Way CW 3<sup>rd</sup>. Crocetin inhibits mRNA expression for tumor necrosis factor-alpha, interleukin-1beta, and inducible nitric oxide synthase in hemorrhagic shock. *JPEN J Parenter Enteral Nutr.* 2006 Jul-Aug;30(4):297-301. (5 pages)
- II. Yang R, Martin L, Woodall C, Thomas AM, Qureshi N, Morrison DC, Van Way CW III. Administration of glutamine following hemorrhagic shock restores cellular energy, reduces cell apoptosis and damage, and increases survival. *JPEN J Parenter Enteral Nutr.* 31:94-100,2007. (7 pages)
- III. Yang R, Tan X, Thomas AM, Qureshi N, Morrison DC, Van Way CW III.: Alanine-Glutamine Dipeptide (AGD) Inhibits Expression of Inflammation-Related Genes in Hemorrhagic Shock. *JPEN Jour Parent Ent Nutr* 31:32-36, 2007. (5 pages)
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## Original Communications

# Crocetin Inhibits mRNA Expression for Tumor Necrosis Factor- $\alpha$ , Interleukin-1 $\beta$ , and Inducible Nitric Oxide Synthase in Hemorrhagic Shock

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**ABSTRACT.** *Background:* Inflammatory factors play an important role in cellular damage after shock and resuscitation. Crocetin, a saffron-derived carotenoid, has been shown to improve postshock recovery of cellular adenosine triphosphate (ATP) and to increase overall survival in an experimental model of hemorrhagic shock. The hypothesis of the present study is that treatment with crocetin at the beginning of resuscitation suppresses subsequent expression of messenger ribonucleic acid (mRNA) for tumor necrosis factor (TNF- $\alpha$ ), interleukin-1 (IL-1 $\beta$ ) and inducible nitric oxide synthase (iNOS). *Methods:* Male Sprague-Dawley rats ( $n = 45$ ,  $350 \pm 30$  g) were randomly assigned to 5 groups of 9 animals each. After anesthesia with isoflurane, the femoral artery and vein were surgically cannulated. Hemorrhagic shock was induced by withdrawing blood through the arterial cannula until the mean arterial pressure (MAP) was 25–30 mm Hg and maintained at the level for 30 minutes with further withdrawals. Resuscitation was carried out by giving 21

mL/kg Ringer's lactate (LR) and returning the shed blood, with or without the initial administration of crocetin (2 mg/kg). Controls were normal (anesthesia only), sham (surgical preparation), and shock (preparation and shock). Rats were killed 30 minutes after completion of resuscitation. Liver samples were collected for reverse transcription-polymerase chain reaction (RT-PCR) analysis of mRNA (TNF- $\alpha$ , IL-1 $\beta$ , iNOS, and  $\beta$ -actin). *Results:* Liver mRNA expression for TNF- $\alpha$ , IL-1 $\beta$ , and iNOS was found in more animals in the shock and shock-plus-resuscitation groups than in the sham control group. The group resuscitated from shock with crocetin had mRNA expression for TNF- $\alpha$ , IL-1 $\beta$ , and iNOS in fewer animals than either of the other shock groups and was no different from the sham control group. *Conclusions:* Crocetin modified the hepatic mRNA expression of cytokines and iNOS in a shock model. This agent continues to show promise as a potential treatment for hemorrhagic shock. (*Journal of Parenteral and Enteral Nutrition* 30:297–301, 2006)

Advances in shock therapy have made it possible to resuscitate all but the most severely injured patients with blood, blood components, and intravenous (IV) fluids. However, the sequelae of shock and resuscitation include widespread tissue and organ injury, which can lead to multiple-organ failure and death. The current focus of resuscitative efforts is on restoration of blood volume and control of hemorrhage, not on prevention of subsequent injury.

Inflammatory factors play an important role in the response to reperfusion and are instrumental in the induction of organ failure after shock. Uncontrolled systemic activation of the inflammatory response may lead to cellular damage and organ dysfunction. Studies have shown that hemorrhagic shock and resuscitation are associated with the activation of several proinflammatory genes in the liver and the development of hepatocellular injury.<sup>1</sup> In addition, hepatic inducible nitric oxide synthase (iNOS) expression is an early molecular

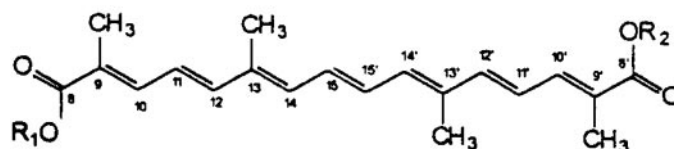


FIGURE 1. Structure diagram of the crocetin molecule. Reprinted from Tarantilis PA, Morjani H, Polissiou M, Manfait M. Inhibition of growth and induction of differentiation of promyelocytic leukemia (HL-60) by carotenoids from *C. Sativus* L. *Anticancer Res.* 1994;14:1913–1918, with permission from the International Institute of Anticancer Research (IIAR).

response to hemorrhagic shock in experimental animals and in injured patients.<sup>2</sup>

Crocetin, a constituent of saffron, has long been used in traditional Chinese medicine as a treatment for many diseases. It is a 16-carbon chain with 8 double bonds and 4 attached methyl groups and a carboxylic acid ( $-\text{COOH}$ ) group at each end of the molecule (Figure 1). The molecular weight is 328. It was reported by Gainer and coworkers<sup>3</sup> to increase oxygen diffusion and to improve tissue oxygenation postresuscitation.<sup>4</sup> It has recently been shown by us that crocetin improves the recovery of cellular ATP and improves survival in the rat model of hemorrhagic shock.<sup>5,6</sup>

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To better understand how crocetin affects the response to hemorrhagic shock, we have investigated the effect of crocetin on the inflammatory response in a sublethal hemorrhagic shock model. The present study has measured mRNA expression in the liver for tumor necrosis factor (TNF- $\alpha$ ), interleukin-1 (IL-1 $\beta$ ), and iNOS. The hypothesis of this study was that crocetin suppresses postshock expression of mRNA for TNF- $\alpha$ , IL-1 $\beta$ , and iNOS.

#### MATERIALS AND METHODS

##### *Rats' Hemorrhagic Shock Model*

Sprague-Dawley male rats ( $n = 45$ ,  $350 \pm 30$  g) were randomly assigned to 5 groups, each group containing 9 animals. Controls were normal controls (anesthetized with isoflurane 1% for 115 minutes), sham controls (surgical preparation, no shock), and shock controls (preparation and shock, no resuscitation). The experimental groups were Ringer's lactate (LR) group (without crocetin) and crocetin group (with crocetin) during resuscitation.

All rats were anesthetized with isoflurane (Baxter Healthcare Corporation, Deerfield, IL) using an anesthesia vaporizer (SurgiVet, Inc. Waukesha, WI) with 100% oxygen. Animals were inducted with 3% isoflurane in an anesthesia chamber and then positioned supine on the table with a nose mask for maintenance of anesthesia. Isoflurane was adjusted from 3% to 1% during the surgical procedure, and then maintained at 1% until the conclusion of the experiment.

Rats were covered with a surgery drape. The temperature under the drape was controlled by a lamp over the table, maintaining it approximately at 28°C during shock and 36°C during resuscitation. This was intended to simulate ambient temperatures during shock and active rewarming during resuscitation.

Vascular access was obtained through a left groin incision. Dissection was carried down to the femoral artery and vein, and both were cannulated using PE50 polyethylene tubing (Becton Dickinson and Company, Franklin Lakes, NJ). The arterial cannula was filled with normal saline containing 100 units of heparin per mL and connected to a solid-state pressure transducer. A digital blood pressure analyzer (Micro-Med, Louisville, KY) was used to monitor pressure continuously. All animals were anticoagulated with heparin (300 U/kg) through the femoral vein cannula after the procedure was completed. At the conclusion of the surgical procedure, the animals were monitored for 5 minutes before shock was initiated.

Hemorrhagic shock was produced by withdrawing blood through the left femoral arterial cannula quickly at first, then more slowly over a 5-minute period, until the mean blood pressure (MAP) stabilized at 25–30 mm Hg. The blood pressure was maintained at that level, with further withdrawals of blood as required for another 30 minutes.

At the start of resuscitation, in the crocetin group, crocetin (ICN Biomedicals Inc, Aurora, OH), dissolved in normal saline (0.2%), was given as a bolus of 2 mg/kg (1 mL/kg). In all animals, resuscitation was carried out by giving LR, 21 mL/kg, followed by return of the shed

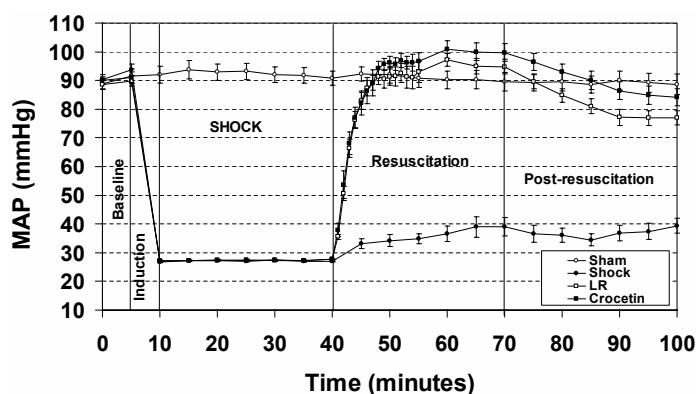


FIGURE 2. Time course of mean arterial pressure (MAP) during shock and resuscitation. Sham control group remained at baseline; shock control, Ringer's lactate (LR), and crocetin groups' MAP fell to 25–30 mm Hg for 30 minutes. LR and crocetin groups recovered to baseline during resuscitation.

blood to the animal. Resuscitation fluid and blood were given over a total of 30 minutes.

Monitoring was continued for 30 minutes after completion of resuscitation. At that point, a midline laparotomy incision was made, and liver biopsies were taken. Samples were frozen in liquid nitrogen immediately and then stored in  $-80^{\circ}\text{C}$  refrigerator for further studies. After that, animals were killed by exsanguination. Figure 2 shows the hemorrhagic shock procedure.

##### *Institutional Approval*

All animal care and experimental procedures were carried out under the protocols of the Animal Care and Use Facility of the University of Missouri–Kansas City, which are in accordance with the guidelines of the American Association for the Accreditation of Laboratory Animal Care. The experimental protocols under which this work was carried out were approved by the Institutional Animal Care and Use Committee of the University of Missouri–Kansas City.

##### *Reverse Transcription–Polymerase Chain Reaction (RT-PCR)*

Total ribonucleic acid (RNA) was extracted from liver samples using the RNeasy Mini Kit (Qiagen Companies, Valencia, CA). The concentration of the RNA was calculated from absorbance at 260 and 280 nm using a spectrophotometer (Beckman, Fullerton, CA). RNA sample concentrations were adjusted to 0.5  $\mu\text{g}/\mu\text{L}$  for reverse transcription-polymerase chain reaction (RT-PCR; TNF- $\alpha$ , IL-1 $\beta$ , iNOS, and  $\beta$ -actin).

RT-PCR was done with the OneStep RT-PCR Kit (Qiagen Companies). The following components were added to the reaction vials: 5  $\mu\text{L}$  5  $\times$  Qiagen OneStep RT-PCR Buffer; 1  $\mu\text{L}$  dNTP Mix (containing 10 mmol/L of each dNTP); 1  $\mu\text{L}$  Qiagen OneStep RT-PCR Enzyme Mix; 1  $\mu\text{M}$  primer (forward and reverse); 1  $\mu\text{g}$  template RNA. The total volume made up to 25  $\mu\text{L}$  with RNase-free water.

Thermal cycler conditions were as follows: (1) reverse transcription:  $50^{\circ}\text{C}$ , 32 minutes; (2) initial PCR

TABLE I  
Oligonucleotide primers for RT-PCR

Protein	Direction	Primer sequence	Size (bp)
TNF- $\alpha$	Forward	5'-AAGTTCCCAAATGGCCTCCCTCTCATC-3'	300
	Reverse	5'-GGAGGTTGACTTCTCTCGGTATGAGA-3'	
IL-1 $\beta$	Forward	5'-AGGCTTCCTTGTGCAAGTGT-3'	230
	Reverse	5'-TGAGTG ACACTGCCTTCCTG-3'	
iNOS	Forward	5'-TCGAGCCCTGGAAGACCCACATCTG-3'	250
	Reverse	5'-GTTGTTCTCTTCCAAGGTGTTG CCTAT-3'	
$\beta$ -Actin	Forward	5'-CAGTCCGCCTAGAAGCATTT-3'	153
	Reverse	5'-GATCATTGCTCCTCCT GAGC-3'	

bp, base pairs; IL-1 $\beta$ , interleukin-1; iNOS, inducible nitric oxide synthase; RT-PCR, reverse transcription-polymerase chain reaction; TNF- $\alpha$ , tumor necrosis factor.

activation step: 95°C, 15 minutes; (3) denaturation: 94°C, 45 seconds; (4) annealing: 62°C, 45 seconds; (5) extension: 72°C, 1 minute; (6) number of cycles: TNF- $\alpha$  was 30 cycles, iNOS was 28 cycles, IL-1 $\beta$  and  $\beta$ -actin were 24 cycles; (7) final extension: 72°C, 10 minutes.

The product of RT-PCR (5  $\mu$ l) plus loaded buffer (1  $\mu$ l) was loaded in a 2% agarose gel and separated by electrophoresis at 87 V for 45 minutes. The gel was stained in 0.5  $\mu$ g/mL ethidium bromide for 30 minutes and then placed under an ultraviolet source to be photographed (Polaroid, Waltham, MA). The molecular sequence and expected size (in base pairs) of RT-PCR products for the various primers are shown in Table I.

The results were expressed as the number of animals in each group that were positive for TNF- $\alpha$ , IL-1 $\beta$ , and iNOS mRNA. The complementary deoxyribonucleic acid (cDNA) electrophoretic bands of all samples were compared with the known positive controls at each locus, recording each sample as being either positive or negative at that locus.

### Statistical Analysis

A 2-way analysis of variance was used to analyze all of the data. The significance level was set at 0.05. Data were further analyzed with Fisher's exact test (<http://www.matforsk.no/ola/fisher.htm>) and Kruskal-Wallis multiple-comparison  $z$ -value test with NCSS software (NCSS, Kaysville, UT).

### RESULTS

A total of 45 rats were used in this study. There were no deaths during the experiments. Figure 2 shows that MAP in the sham group did not change during the experiment. MAP in the shock group, LR group, and crocetin group fell to the same level during shock. Shock controls recovered somewhat after shock but continued to show statistically lower MAP than the others until the end of the experiment. The MAP in

both LR and crocetin groups recovered to baseline within 10 minutes of beginning fluid resuscitation and exceeded baseline values during resuscitation. After resuscitation, the MAP decreased gradually to a little lower than baseline, but the difference was not statistically significant.

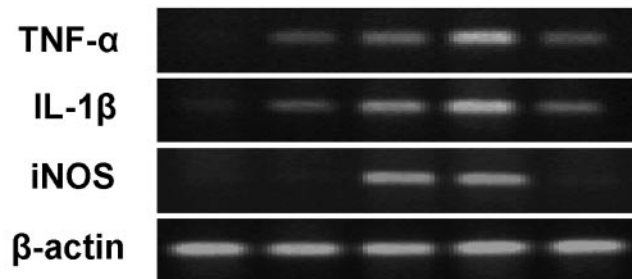
Table II shows rats' weight, surgery time, and blood removal during the experiment. There were no differences among groups for weight, surgery time, and blood withdrawal.

Figure 3 shows mRNA expression for a representative animal in each group. A strong  $\beta$ -actin band indicated that adequate RNA was obtained from all samples.

Figure 4 shows the number of animals showing mRNA expression. Normal control rats were not positive for TNF- $\alpha$  and IL-1 $\beta$  mRNA. Only 2–3 of the sham control were positive for TNF- $\alpha$ , IL-1 $\beta$ , or iNOS mRNA. In the LR group, and in the shock controls, most animals showed expression of mRNA for TNF- $\alpha$ , IL-1 $\beta$ , and iNOS. Only 3 rats in the crocetin-resuscitated group were positive for TNF- $\alpha$ , IL-1 $\beta$ , and iNOS, and the crocetin group was not significantly different from the sham control group. The crocetin group was positive for IL-1 $\beta$  and iNOS in significantly fewer animals than the LR group ( $p < .05$ ).

### DISCUSSION

Our results suggested that hemorrhagic shock and resuscitation with LR are associated with appearance of hepatic mRNA for TNF- $\alpha$ , IL-1 $\beta$ , and iNOS. Crocetin-resuscitated animals showed an mRNA response



Experiment Normal Sham Shock LR Crocetin

FIGURE 3. TNF- $\alpha$ , IL-1 $\beta$ , iNOS, and  $\beta$ -actin mRNA expression. Taken from a representative rat in different experimental groups.

TABLE II  
Rats' weight, surgery time and blood withdrawing

	Sham	Shock	LR	Crocetin
Weight (g)	364 $\pm$ 5.6	358 $\pm$ 7.0	377 $\pm$ 15	356 $\pm$ 7.1
Surgery time (min)	8.0 $\pm$ 0.2	7.2 $\pm$ 0.5	7.1 $\pm$ 0.4	7.3 $\pm$ 0.4
Blood withdrawing (mL)		10.0 $\pm$ 0.7	11.0 $\pm$ 0.6	10.0 $\pm$ 0.5



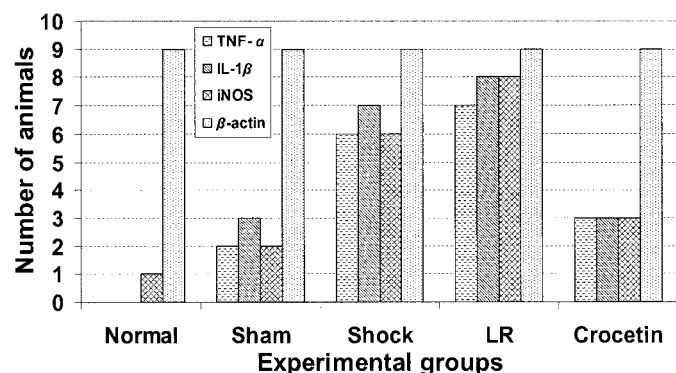


FIGURE 4. Number of animals' mRNA expressed for TNF- $\alpha$ , IL-1 $\beta$ , iNOS, and  $\beta$ -actin in different experimental groups.

no different from sham controls. The results indicate that proinflammatory gene activation occurs in the liver quite early after shock and resuscitation. The experimental hypothesis, that crocetin would suppress proinflammatory gene activation, is supported by the results of the experiments.

Inflammatory cytokines have been implicated in tissue damage and mortality in hemorrhagic shock.<sup>7,8</sup> Resuscitation from hemorrhagic shock triggers an inflammatory response characterized by up-regulation of cytokine expression.<sup>9</sup> TNF- $\alpha$  and IL-1 $\beta$  have been reported to be derived predominantly from activated macrophages and act *via* specific cell-membrane-bound receptors.<sup>10,11</sup> In a review, Angele and Faist<sup>12</sup> indicated that increased plasma levels of proinflammatory cytokines occur after trauma and hemorrhage, and these cytokines can act to depress macrophage function. *In vivo* administration of TNF- $\alpha$  and IL-1 $\beta$  induces a shocklike syndrome similar to that observed after severe blood loss and sepsis. It has been suggested that these cytokines may play a role in initiating the cascade of events leading to the development of multiple organ dysfunction after severe hemorrhagic shock.<sup>12</sup>

Although not a cytokine, iNOS is significantly up-regulated after hemorrhagic shock and resuscitation. It is thought to be one of the major contributors to hepatic injury caused by severe hemorrhagic shock.<sup>2,13</sup> The mechanism of up-regulation is unclear, but it may be initiated by hypoxia or by cytokines. Our results confirm that iNOS is up-regulated after shock and resuscitation.<sup>14</sup> Several studies have suggested that excessive NO production contributes to the organ injury associated with hemorrhagic shock and resuscitation.<sup>14–18</sup> Induction of iNOS contributes to the initiation of a complex inflammatory response, including activation of signaling proteins and cytokine expression, ultimately leading to morphologic and functional injury.

Resuscitation with crocetin reduced liver TNF- $\alpha$  mRNA expression and inhibited IL-1 $\beta$  and iNOS mRNA expression significantly compared with resuscitation with LR alone (Figure 4). These results confirm our hypothesis that crocetin suppresses postshock overexpression of mRNA for TNF- $\alpha$ , IL-1 $\beta$ , and iNOS. Apparently, crocetin modified the expression of genes controlling cytokine and iNOS in the liver.

Our previous experiments have shown that crocetin increased the survival of rats subjected to lethal hem-

orrhagic shock. Crocetin also accelerates the return of cellular energy stores to normal after hemorrhagic shock and produces a marked diminution in the extent of apoptosis postshock. The mechanism of action probably involves prevention of mitochondrial damage.<sup>5,6</sup> Crocetin is thought to increase oxygen transport in the plasma and may enhance oxygen supply at the cellular level.<sup>3,4</sup> Other investigators have found that crocetin has anti-inflammatory activity, is an antioxidant,<sup>19–22</sup> and protects against hepatotoxic agents.<sup>21,23</sup>

## CONCLUSION

This agent continues to have potential for treatment of hemorrhagic shock. The development of new pharmacologic strategies to minimize late tissue and organ damage will greatly improve the clinical treatment of hemorrhagic shock. Subsequent investigations will determine the mechanisms and whether action is primary or secondary to increased oxygen transport.

## ACKNOWLEDGMENTS

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## Original Communications

# Administration of Glutamine After Hemorrhagic Shock Restores Cellular Energy, Reduces Cell Apoptosis and Damage, and Increases Survival

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**ABSTRACT.** *Background:* Hemorrhagic shock causes a rapid depletion of adenosine triphosphate (ATP) and an increase of the terminal metabolite xanthine. Free radicals generated from xanthine oxidase play a major role in cell injury. Programmed cell death, apoptosis, is a major pathway causing reperfusion injury. During apoptosis, cytosolic cytochrome-c is released from damaged mitochondria, and it further initiates activation of apoptosis as evidenced by the appearance of caspase-3. The bcl-2 protein serves as an antiapoptosis found on the mitochondrial membrane. Glutamine has been known as a conditionally essential nutrient and seems to have beneficial effects in critically ill patients. The hypothesis of the present study is that glutamine administered during resuscitation following hemorrhagic shock would restore the depletion of hepatic ATP, reduce cellular apoptosis, and increase survival. *Methods:* Male Sprague-Dawley rats were randomly assigned to 3 groups for resuscitation after the

same pattern of hemorrhagic shock: Ringer's lactate (LR 21 mL/kg); Alanine-glycine (LR with alanine 0.15 g/kg and glycine 0.18 g/kg); and glutamine (LR with glutamine 0.3 g/kg). Hepatic ATP and xanthine was measured at different time periods. Hepatic apoptosis was measured and the levels of cytosolic cytochrome-c, caspase-3 and bcl-2 were analyzed. Another group of rats were used for survival study. *Results:* Glutamine administered during resuscitation following hemorrhagic shock partially restored the depletion of hepatic ATP, reduced cellular apoptosis, and increased survival. *Conclusions:* Glutamine administration during resuscitation significantly protected the liver from tissue damage caused by hemorrhagic shock. Glutamine supplementation may offer opportunities for therapeutic intervention during and after shock. (*Journal of Parenteral and Enteral Nutrition* 31:94–100, 2007)

Hemorrhagic shock has a profound effect on cellular energy stores. Previous studies have shown that there is a severe decrease in cellular adenosine triphosphate (ATP) levels after hemorrhagic shock.<sup>1–4</sup> In particular, decreased hepatic ATP levels after hemorrhage have been correlated with intracellular edema and mitochondrial damage, which lead to cell injury and cell death.<sup>5–8</sup>

Glutamine is a precursor of ATP. Although it can be synthesized in the body, it is depleted in stress and is thus considered to be a conditionally essential nutrient. The present study was undertaken to determine if provision of exogenous glutamine would facilitate the resynthesis and recovery of ATP. A sublethal hemorrhagic shock rat model was used. Glutamine was administered during resuscitation. Alanine and glycine, an isocaloric mixture of amino acids, was provided as an amino acid control. The hypothesis was that glutamine, administered during resuscitation after hemorrhagic shock, would restore the depletion of hepatic ATP, reduce cellular apoptosis, and increase

survival. Hepatic ATP and its metabolite xanthine were measured. Apoptosis, cytosolic cytochrome-c, caspase 3, and bcl-2 protein were assayed. Furthermore, a survival study was performed to compare resuscitation with or without glutamine.

## MATERIALS AND METHODS

### Hemorrhagic Shock Model

Animals were maintained in the Animal Care Facility of the University of Missouri–Kansas City. Animals were kept on 12:12-hour light-dark cycle with free access to food and water. Male Sprague-Dawley rats (300–350 g) were randomly assigned to 3 groups (Ringer's lactate [LR], alanine-glycine and glutamine), each of which was subjected to hemorrhagic shock in a similar manner. Resuscitation was with LR and return of shed blood. In the LR group, rats received LR 21 mL/kg, with no other pharmacologic treatment. In the alanine-glycine group, rats received LR 21 mL/kg, with alanine 0.15 g/kg and glycine 0.18 g/kg. In the glutamine group, rats received LR 21 mL/kg, with glutamine 0.3 g/kg.

All rats were anesthetized with isoflurane (Baxter Healthcare Corporation, Deerfield, IL) using an anesthesia vaporizer (SurgiVet, Inc. Waukesha, WI) with 100% oxygen while breathing spontaneously. Isoflu-

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rane was adjusted from 3% to 1% after induction and then maintained at 1% until the conclusion of the experiment.

Rats were covered with a surgery drape. The temperature under the drape was controlled by a lamp over the table, maintaining it approximately at 28°C during shock and 36°C during resuscitation. Vascular access was obtained over the left neck incision. Dissection was carried down to the carotid artery and jugular vein, and both were cannulated using PE50 polyethylene tubing (Becton Dickinson and Company, Sparks, MD). All animals were anticoagulated with heparin (300 units/kg) through the venous cannula after the procedure was completed. A digital blood pressure analyzer (Micro-Med, Louisville, KY) was used to monitor pressure continuously. Hemorrhagic shock was produced by withdrawing blood through the left carotid arterial cannula over a 5-minute period, until the mean arterial pressure (MAP) stabilized at 30–35 mm Hg. The MAP was maintained at that level, with further withdrawals of blood as required for 30 minutes. MAP was monitored continuously and recorded every 5 minutes.

Resuscitation was carried out by giving LR (21 mL/kg), with or without alanine-glycine or glutamine dissolved in LR, as outlined above, followed by return of the shed blood. Resuscitation fluid and blood were given slowly over a total of 30 minutes.

Liver biopsies by freeze-clamping were carried out through a small midline laparotomy incision at baseline, at the end of the shock period, and 30 minutes and 60 minutes after completion of resuscitation.<sup>2</sup> Separate groups of 7–8 animals were harvested at 24 and 48 hours after the shock period. In these animals, no laparotomy was done initially. After resuscitation, they were decannulated and allowed to recover. At the later time, general anesthesia was induced, a midline laparotomy incision was made, liver biopsies were taken, and the animals were killed. All liver samples were frozen in liquid nitrogen immediately and then stored at –80°C until further studies were done.

#### *High-Performance Liquid Chromatography*

Separation of ATP and its metabolites, including xanthine, was carried out using gradient high-performance liquid chromatography (Beckman Instruments, Fullerton, CA) using a Supelcosil LC-18-T reverse-phase analytical column (Supelco, Bellefonte, PA). Integration of peak areas was performed on the HP3392A electronic integrator (Hewlett-Packard, Monterey, CA). The Beckman Gold System software was used for both system control, output display, and data processing.<sup>2,4</sup>

#### *Apoptosis*

Liver cytosolic extract was analyzed for apoptosis, cytochrome-c, caspase-3, and bcl-2 by using an enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's recommendation. Apoptosis was assayed by using a Cell Death Detection ELISA-PLUS kit (Roche Applied Science, Indianapolis, IN). Cytochrome-c was analyzed by using an assay kit (MBL

International, Woburn, MA). Caspase-3 was assayed by using a Caspase-3 Protease Assay kit (BioSource International, Inc, Camarillo, CA). Bcl-2 was analyzed by using a bcl-2 ELISA kit (Oncogene Research Products, San Diego, CA).

Briefly, standards or experimental samples were added to microplates coated with the monoclonal antibody of interest, followed by the addition of a biotinylated second antibody. After the first incubation and washing, streptavidin-peroxidase (enzyme) was added and conjugated. After second incubation and washing, substrate solution was added and incubated. The determination was assessed by absorbance at 450 nm using a microtiter plate reader (Dynatech Laboratories, Chantilly, VA).

#### *Survival*

For survival studies, separate groups of rats were subjected to hemorrhagic shock at 30 or 90 minutes, followed by resuscitation with LR (21 mL/kg), with or without glutamine (0.3 g/kg) dissolved in LR, plus return of shed blood. Animals were monitored for 72 hours.

#### *Statistical Analysis*

Data were expressed as mean  $\pm$  SEM and were analyzed using Microsoft Excel (Microsoft Corp, Redmond, WA). Data were further analyzed by using Kruskal-Wallis multiple-comparison z-value test (NCSS, Kaysville, UT). One-tail analysis of variance was used to compare survival rates (Microsoft Excel). Data were further analyzed with Fisher's exact test (<http://www.matforsk.no/ola/fisher.htm>). The significance level was .05 for all analyses.

#### *Institutional Approval*

All animal care and experimental procedures were carried out according to the Guidelines of the Laboratory Animal Center of the University of Missouri–Kansas City, which is accredited by the American Association for the Accreditation of Laboratory Animal Care. The experimental protocols under which this work was carried out were approved by the Institutional Animal Care and Use Committee of the University of Missouri–Kansas City.

### RESULTS

#### *Glutamine Restores Cellular Energy After Hemorrhagic Shock*

The baseline values of ATP and xanthine were similar in the different groups before the initiation of shock. Compared with the baseline, as can be seen in Figure 1, hepatic ATP levels were significantly reduced after shock. The groups receiving LR alone and LR plus alanine-glycine both showed ATP depletion at 30 and 60 minutes postresuscitation, and ATP levels remained depressed at 24 hours. Hepatic ATP levels in the group that received glutamine rose toward baseline levels more quickly at 30 minutes postresuscitation, and by 60 minutes they were not significantly different

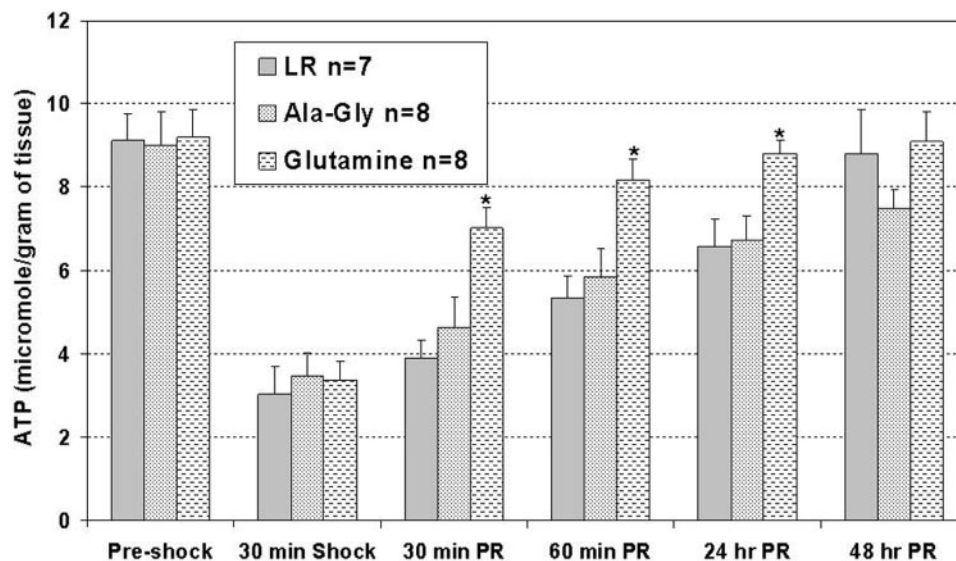


FIGURE 1. Hepatic adenosine triphosphate (ATP) concentrations in 3 groups of animals, measured at time intervals before shock, after 30 minutes of shock, and postresuscitation (30 minutes, 60 minutes, 24 hours, and 48 hours; PR). \*  $p < .05$  (Glutamine vs LR [Ringer's lactate] and Ala-Gly [alanine-glycine]).

from baseline levels. Xanthine levels were significantly increased after shock in all groups (Figure 2). At 60 minutes postresuscitation, hepatic xanthine levels were lower in the glutamine group than in the LR and alanine-glycine groups.

#### Glutamine Administration Diminishes Tissue Apoptosis

Apoptosis was increased at 24 and 48 hours in the LR and alanine-glycine groups, whereas the glutamine group showed no significant increase above baseline (Figure 3).

Cytosolic cytochrome-c was present 24 and 48 hours postresuscitation. The level was significantly less in the glutamine groups compared with the LR and alanine-glycine groups (Figure 4).

Hepatic caspase-3 activation was observed at 24 and 48 hours in the LR and alanine-glycine groups. It was significantly less in the glutamine group (Figure 5).

Liver bcl-2 protein was diminished at 24 and 48 hours postresuscitation. However, bcl-2 levels in the glutamine group was significantly higher than in the 2 control groups (Figure 6).

#### Glutamine Improves Survival After Hemorrhagic Shock

In separate studies of survival, the animal preparation was modified so that either a 30-minute or a 90-minute period of shock was used before resuscitation. Animals were followed for 3 days. Administration of glutamine during resuscitation enhanced survival

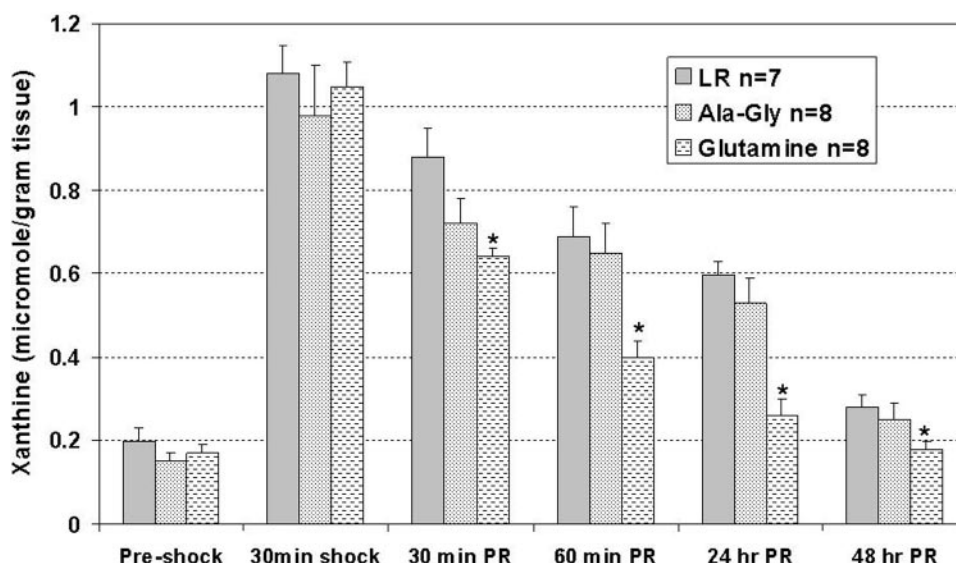


FIGURE 2. Hepatic xanthine concentrations in 3 groups of animals, measured at time intervals before shock, after 30 minutes of shock, and after resuscitation (30 minutes, 60 minutes, 24 hours, and 48 hours; PR). \*  $p < .05$  (glutamine vs LR and Ala-Gly).



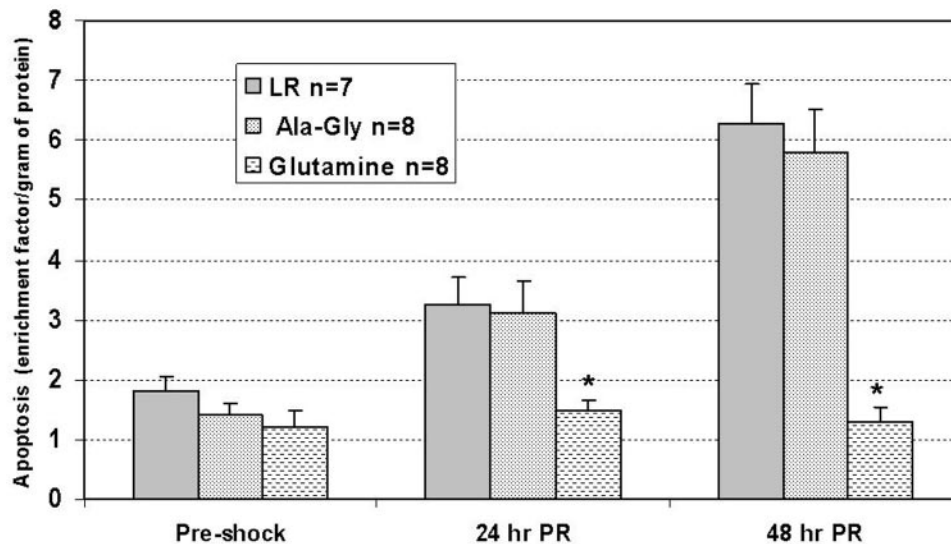


FIGURE 3. Liver cell apoptosis in 3 groups of animals, measured at preshock and postresuscitation (PR) 24 and 48 hours. \*  $p < .05$  (Glutamine vs LR and Ala-gly).

after the 90-minute shock period significantly compared with resuscitation with LR alone (Figure 7).

#### DISCUSSION

The data presented support the hypothesis that glutamine, when administrated during resuscitation after hemorrhagic shock, will restore hepatic ATP. Work in our laboratory has studied the effect of shock on cellular nucleotide levels.<sup>9</sup> During normal metabolism, ATP is metabolized to adenosine, inosine, and finally to hypoxanthine and xanthine.<sup>2,9</sup> Hemorrhagic shock has been shown to cause depletion of ATP and corresponding elevation of all ATP metabolites.<sup>9–11</sup> Xanthine accumulates during ischemia. Then, when oxygenation is restored, the action of xanthine oxidase on xanthine causes a burst of oxygen free radicals, which in turn

lead to cause cellular injury.<sup>12–15</sup> ATP levels are reconstituted relatively slowly after even a brief period of shock, returning to baseline only after 24–48 hours, whereas elevated levels of xanthine may persist beyond that time.<sup>2,4,7,12</sup>

In the present study, all groups of animals showed the expected reduction in liver ATP and increase in xanthine after 30 minutes of hemorrhagic shock. In the groups receiving LR and alanine-glycine, these metabolic alterations persisted, for up to 48 hours postresuscitation. In contrast, animals treated with glutamine during resuscitation showed return of ATP tissue levels to preshock levels in about 2 hours. Some elevation of xanthine was seen even at 48 hours, but it was less than that seen in the control groups.

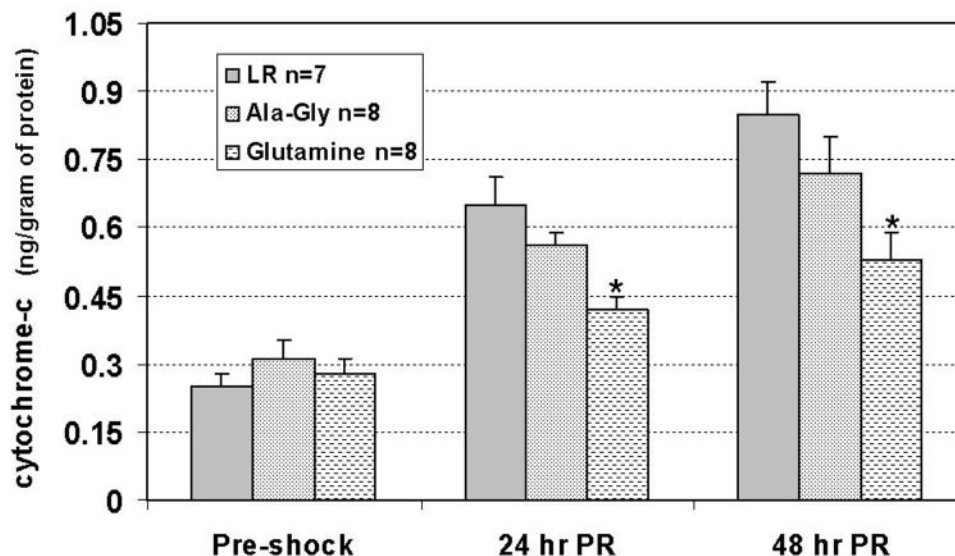


FIGURE 4. Liver cytosolic cytochrome-c levels in 3 groups of animals, measured at preshock and postresuscitation (PR) 24 and 48 hours. \*  $p < .05$  (Glutamine vs LR and Ala-Gly).

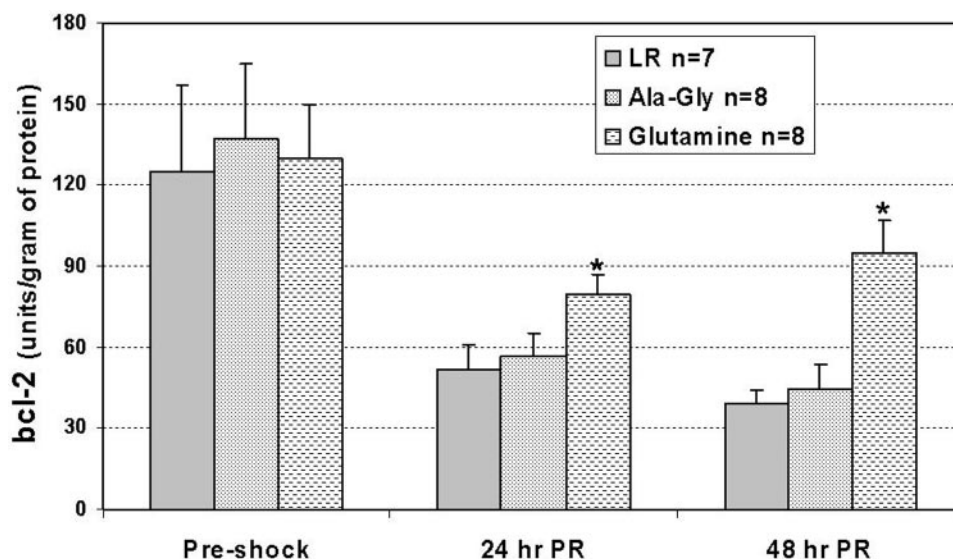


FIGURE 5. Bcl-2 activity in 3 groups of animals, measured at preshock and postresuscitation (PR) 24 and 48 hours. \* $p < .05$  (Glutamine vs LR and Ala-Gly).

It is highly likely that apoptosis is a major mechanism by which organ damage results from reperfusion injury.<sup>16,17</sup> Apoptosis is initiated by mitochondrial damage. Cytochrome-c is released into the cellular cytoplasm from damaged mitochondria. Its release is a signal to initiate activation of the apoptotic pathway. As shown in Figure 4, there was a marked increase in free cytochrome-c detectable in the cytoplasmic fraction of hepatic tissues compared with baseline at 24 and 48 hours in the LR and alanine-glycine groups. In the glutamine group, by contrast, cytosolic cytochrome-c levels remained near baseline.

The appearance of caspase-3 is generally accepted as evidence that the apoptotic pathway has been activated and will proceed to apoptotic cell death.<sup>18</sup> As shown in Figure 5, there was a marked increase in hepatic caspase-3 compared with baseline at 24 and 48

hours after a period of shock in the LR and alanine-glycine groups. In the glutamine group, caspase-3 levels remained near baseline.

The bcl-2 protein, a constituent of the mitochondrial membrane, is thought to serve a protective function for the mitochondrion against ischemic damage. Previous research has established that levels of bcl-2 protein are severely depressed after shock and resuscitation.<sup>19-21</sup> As shown in Figure 6, levels of bcl-2 were markedly depressed at 24 and 48 hours in the LR and alanine-glycine groups but significantly less so in the glutamine group. Treatment with glutamine appeared to contribute to recovery of hepatic bcl-2 levels after shock.

Ultimately, any effective therapeutic agent must improve survival. Importantly, the administration of glutamine during resuscitation significantly enhanced survival in this shock model.

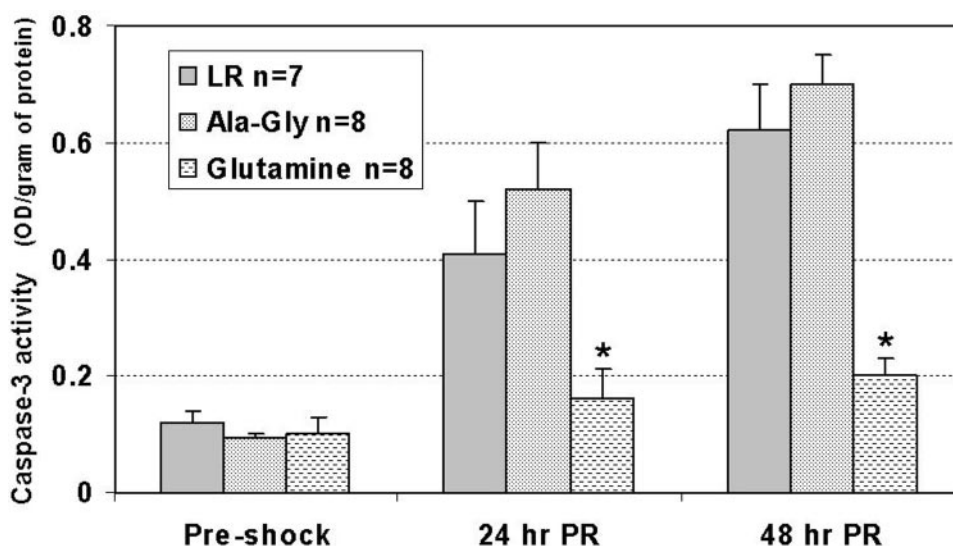


FIGURE 6. Caspase-3 levels in 3 groups of animals, measured preshock and postresuscitation (PR) 24 and 48 hours. \* $p < .05$  (Glutamine vs LR and Ala-Gly).

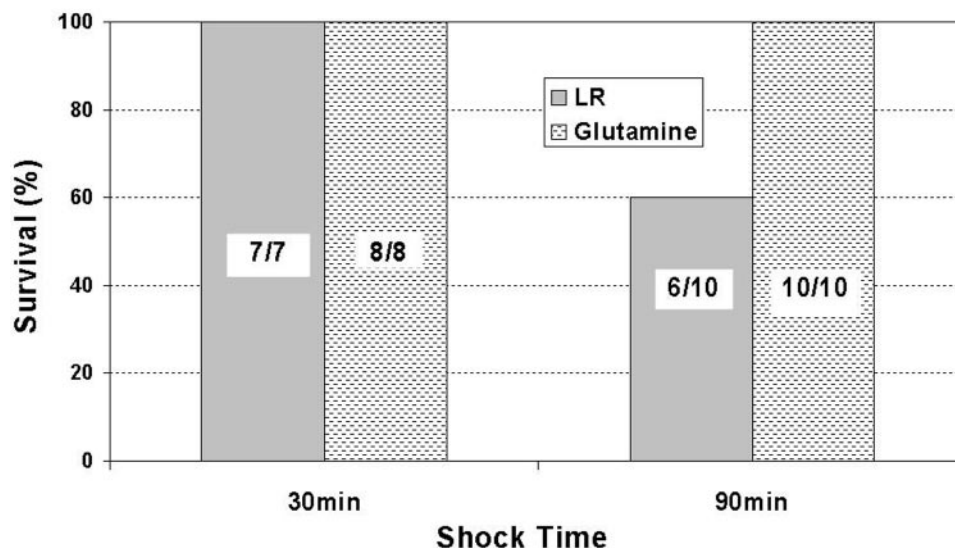


FIGURE 7. Survival rates at 72 hours after shock periods of 30 minutes and 90 minutes compares Ringer's lactate (LR) alone to LR plus glutamine. \*  $p < .05$  (Glutamine vs LR).

In summary, glutamine is a precursor of ATP synthesis. In stress states, it may be present in the body in insufficient quantities. During conditions of stress, glutamine levels have been documented to decrease dramatically in blood and extracellular fluid, and these conditions are reported to persist throughout recovery.<sup>22,23</sup> Glutamine supplementation has been advocated for patients under stress caused by infection, recent surgery, or traumatic injury.<sup>24–26</sup> Furthermore, glutamine appears to be an immunomodulator and has been used in immune-enhancing nutrition formulas.<sup>27,28</sup> Work from our laboratory has shown that administration of the alanine-glutamine dipeptide, a glutamine source, can suppress messenger RNA (ribonucleic acid) for the cytokines tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-1 $\beta$ , and for inducible NO synthetase (iNOS).<sup>29</sup> The mechanism by which glutamine exerts these effects is not understood, although there is recent published evidence that it may inhibit NF $\kappa$ B activation through suppression of ubiquitination of I $\kappa$ B, thus preventing its degradation by the proteasome.<sup>30–33</sup> Ziegler et al<sup>34</sup> and Wischmeyer et al<sup>35</sup> have postulated that it exerts a protective effect by stimulating the production of heat shock protein (HSP)-70.

#### CONCLUSIONS

Glutamine supplementation significantly protected the liver from tissue damage caused by hemorrhagic shock. Glutamine administration during resuscitation is associated with more rapid return of cellular energy stores toward normal levels. Agents such as glutamine seem to favorably modulate the inflammatory response to shock and resuscitation and prevent cellular damage. Use of such agents may diminish the postshock systemic inflammatory reaction and thus lower the risk of organ failure and subsequent death. Glutamine supplementation may help to protect cellular energy stores in the stressed organism and may offer oppor-

tunities for therapeutic intervention during and after stress.

#### ACKNOWLEDGMENTS

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## Original Communications

### Alanine-Glutamine Dipeptide (AGD) Inhibits Expression of Inflammation-Related Genes in Hemorrhagic Shock

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**ABSTRACT.** *Background:* Inflammatory factors play an important role in the production of cellular damage after shock and reperfusion. Glutamine has been used to modulate the inflammatory response. Alanine-glutamine dipeptide (AGD) is a glutamine source. The hypothesis of the present study is that AGD given during resuscitation will suppress postshock expression of messenger ribonucleic acid (mRNA) for tumor necrosis factor (TNF- $\alpha$ ), interleukin-1 (IL-1 $\beta$ ) and inducible nitric oxide synthase (iNOS). *Methods:* Male Sprague-Dawley rats (n = 74, 350 g  $\pm$  30 g) were randomly assigned to 5 groups. Under isoflurane anesthesia, the femoral artery and vein were cannulated. Hemorrhagic shock was induced by withdrawing blood through the arterial cannula until the mean arterial pressure (MAP) was 25–30 mm Hg and maintained at the level for 30 minutes with further withdrawals. Resuscitation was carried out by giving 21

mL/kg Ringer's lactate (LR) with or without the administration of AGD (936 mg/kg) and returning the shed blood. Controls were normal (anesthesia only), sham (surgical preparation), and shock (preparation and shock). Rats (n = 45, 9 per group) were killed 30 minutes after completion of resuscitation. Liver samples were collected, and total RNA was isolated for reverse transcription–polymerase chain reaction analysis of mRNA (TNF- $\alpha$ , IL-1 $\beta$ , iNOS, and  $\beta$ -actin). *Results:* MAP recovered more quickly in the AGD group than in the LR group. Increased expression of liver mRNA for TNF- $\alpha$ , IL-1 $\beta$ , and iNOS was seen after hemorrhagic shock and resuscitation. AGD treatment significantly reduced mRNA expression for all 3. *Conclusions:* AGD modified the expression of genes controlling cytokines and iNOS in the liver. This agent is a potential treatment for hemorrhagic shock. (*Journal of Parenteral and Enteral Nutrition* 31:32–36, 2007)

Inflammatory factors play an important role in the host response to ischemic injuries and are probably instrumental in the induction of organ failure after hemorrhagic shock. A widely held view is that uncontrolled systemic activation of the inflammatory response leads to cellular damage and organ dysfunction. Studies have shown that hemorrhagic shock and resuscitation is associated with the activation of several proinflammatory genes in the liver and development of hepatocellular injury.<sup>1</sup> In addition, hepatic inducible nitric oxide synthase (iNOS) expression is an early molecular response to hemorrhagic shock in experimental animals and has also been reported in injured patients.<sup>2</sup>

Alanine-glutamine dipeptide (AGD), clinically available in Europe, is rapidly hydrolyzed to alanine and glutamine after IV administration. Unlike glutamine, it is stable in solution and is available as a concentrated solution. AGD, like glutamine, seems to have beneficial effects in critical illness. In studies of critically ill patients, AGD-supplemented parenteral nutrition (PN) has been shown to reduce the length of stay

in the intensive care unit and to improve long-term survival.<sup>3,4</sup> Glutamine has also been shown to increase survival in an animal model of hemorrhagic shock.<sup>5</sup>

To better understand how AGD presumably affects the response to hemorrhagic shock, we have investigated its effect on the cytokine response in a sublethal hemorrhagic shock model. The present study has measured mRNA expression in the liver for tumor necrosis factor (TNF- $\alpha$ ), interleukin 1 (IL-1 $\beta$ ), and iNOS. Our hypothesis was that AGD suppresses postshock expression of mRNA for TNF- $\alpha$ , IL-1 $\beta$ , and iNOS.

#### MATERIALS AND METHODS

##### *Hemorrhagic Shock Model*

A total of 74 rats were used in this study, of which 45 rats were used in mRNA studies, and 29 used to provide additional hemodynamic data. Sprague-Dawley male rats (n = 45, 350 g  $\pm$  30 g) were randomly assigned to 5 groups to study mRNA expression in the liver. Groups of 9 were: normal (anesthetized with isoflurane 1% for 115 minutes), sham controls (surgical preparation, no shock), shock controls (preparation and shock, no resuscitation), LR (resuscitation with Ringer's lactate, without AGD), and AGD (resuscitation with AGD). AGD (Fresenius Kabi, Graz, Austria), was available as a 20% concentrated solution for infusion, of which 100 mL contained N(2)-L-alanyl-D-glu-

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tamine 20 g (D-alanine 8.20 g, D-glutamine 13.46 g). An additional 29 animals were studied to determine the hemodynamic response to resuscitation with LR alone ( $n = 14$ ) or LR plus AGD ( $n = 15$ ).

All rats were anesthetized with isoflurane (Baxter Healthcare Corporation, Deerfield, IL) using an anesthesia vaporizer (SurgiVet, Inc, Waukesha, WI) with 100% oxygen connected to a glass chamber. Animals were induced with 3% isoflurane in the chamber and then positioned supine on the table, using a nose mask for maintenance of anesthesia. Isoflurane was adjusted from 3% to 1% after induction and then maintained at 1% until the conclusion of the experiment.

Rats were covered with a surgery drape. The temperature under the drape was controlled by a lamp over the table, maintaining it at 28°C during shock and 36°C during resuscitation.

Vascular access was obtained surgically. An incision was made over the left groin. Dissection was carried down to the femoral artery and vein, and both were cannulated using PE50 polyethylene tubing (Becton Dickinson and Company, Franklin Lakes, NJ). The arterial cannula was filled with normal saline containing 100 units of heparin per mL and connected to a solid-state pressure transducer. A digital blood pressure analyzer (Micro-Med, Louisville, KY) was used to monitor pressure continuously. All animals were anticoagulated with heparin (300 U/kg) through the femoral vein cannula after the procedure was completed. At the conclusion of the surgical procedure, the animals were monitored for 5 minutes before shock was initiated.

Hemorrhagic shock was produced by withdrawing blood through the left femoral arterial cannula quickly at first, and then more slowly, over a 5-minute period, until the mean arterial blood pressure (MAP) stabilized at 25–30 mm Hg. The blood pressure was maintained at that level with further withdrawals of blood as required for 30 minutes. Blood pressure was monitored continuously and recorded every 5 minutes.

Resuscitation was carried out by giving LR (21 mL/kg), with or without AGD (936 mg/kg, equivalent to glutamine 630 mg/kg) dissolved in LR to a total volume of 21 mL/kg, followed by return of the shed blood to the animal. Resuscitation fluid and blood were given slowly over a total of 30 minutes, administered by using an electric syringe pump (Harvard Apparatus, Holliston, MA). Blood pressure was monitored and recorded every minute during the first 15 minutes of resuscitation.

Monitoring was continued for 30 minutes after completion of resuscitation. At that point, each group containing 9 animals, a total of 45 rats, was harvested. A midline laparotomy incision was made, and liver biopsies were taken. Samples were frozen in liquid nitrogen immediately, and then stored in a –80°C refrigerator for further studies. After that, animals were killed by exsanguination.

In the 29 animals studied for hemodynamic response, blood pressure was monitored until after resuscitation, and animals were killed at differing times up until 90 minutes postresuscitation.

### *Institutional Approval*

All animal care and experimental procedures were carried out according to the Guidelines of the Laboratory Animal Center of the University of Missouri–Kansas City, which is a facility accredited by the American Association for the Accreditation of Laboratory Animal Care. The experimental protocols under which this work was carried out were approved by the Institutional Animal Care and Use Committee of the University of Missouri–Kansas City.

### *Reverse Transcription–Polymerase Chain Reaction (RT-PCR)*

Total RNA was extracted from liver samples using the RNeasy Mini Kit (Qiagen Companies, Valencia, CA). RNA concentration was calculated from absorbance at 260 and 280 nm using a spectrophotometer (Beckman, Fullerton, CA). RNA sample concentrations were adjusted to 0.5  $\mu\text{g}/\mu\text{L}$  for RT-PCR (TNF- $\alpha$ , IL-1 $\beta$ , iNOS, and  $\beta$ -actin).

RT-PCR was done with the OneStep RT-PCR Kit (Qiagen Companies). The following components were added to the reaction vials: 5  $\mu\text{L}$  5  $\times$  Qiagen OneStep RT-PCR Buffer; 1  $\mu\text{L}$  dNTP Mix (containing 10 mmol/L of each dNTP); 1  $\mu\text{L}$  Qiagen OneStep RT-PCR Enzyme Mix; 1  $\mu\text{M}$  primer (forward and reverse); 1  $\mu\text{g}$  template RNA. The total volume was 25  $\mu\text{L}$  mixed in RNease-free water.

Thermal cycler conditions were as follows: (1) reverse transcription: 50°C, 32 minutes; (2) initial PCR activation step: 95°C, 15 minutes; (3) denaturation: 94°C, 45 seconds; (4) annealing: 62°C, 45 seconds; (5) extension: 72°C, 1 minute; (6) number of cycles: TNF- $\alpha$  was 30 cycles, iNOS was 28 cycles; IL-1 $\beta$  and  $\beta$ -actin were 24 cycles; (7) final extension: 72°C, 10 minutes.

The product of RT-PCR (5  $\mu\text{L}$ ) plus loading buffer (1  $\mu\text{L}$ ) was applied to the top of a 2% agarose gel and separated by electrophoresis at 87 V for 45 minutes. The gel was stained in 0.5  $\mu\text{g}/\text{mL}$  ethidium bromide for 30 minutes and then placed under an ultraviolet source to be photographed (Polaroid, Waltham, MA). The molecular sequence and expected size (in bp) of RT-PCR products for the various primers are shown in Table I.

The results are expressed as the number of animals in each group which were positive for detectable expression of TNF- $\alpha$ , IL-1 $\beta$ , and iNOS mRNA. The cDNA electrophoretic bands of all samples were compared with the known positive controls at each locus, recording each sample as being either positive or negative at that locus.

### *Statistical Analysis*

Data on hemodynamics are expressed as mean  $\pm$  SEM. Data were analyzed using Microsoft Excel. Student's *t*-test was used to compare hemodynamic measurements for the AGD group and the LR group. A significance level was set at .05.

A 2-way analysis of variance was used to analyze all of the gene expression data. The significance level was set at .05. Data were further analyzed with Fisher's

TABLE I  
Oligonucleotide primers for reverse transcription-polymerase chain reaction (RT-PCR)

Protein	Direction	Primer sequence	Size (bp)
TNF- $\alpha$	Forward	5'-AAGTTCCCAAATGGCCTCCCTCTCATC-3'	300
	Reverse	5'-GGAGGTGACTTTCTCCTGGTATGAGA-3'	
IL-1 $\beta$	Forward	5'-AGGCTTCCTTGTGCAAGTGT-3'	230
	Reverse	5'-TGAGTG ACACTGCCTTCCTG-3'	
iNOS	Forward	5'-TCGAGCCCTGGAAGACCCACATCTG-3'	250
	Reverse	5'-GTTGTTCTCTTCCAAGGTGTTTGCCTTAT-3'	
$\beta$ -actin	Forward	5'-CAGTCCGCCTAGAAGCATTT-3'	153
	Reverse	5'-GATCATTGCTCCTCCTGAGC-3'	

IL-1 $\beta$ , interleukin-1; iNOS, inducible nitric oxide synthase; TNF- $\alpha$ , tumor necrosis factor.

exact test (<http://www.matforsk.no/ola/fisher.htm>) and Kruskal-Wallis multiple-comparison z-value test (NCSS, Kaysville, UT).<sup>6</sup>

## RESULTS

There were no differences of the amount of blood withdrawn among shock, LR, and AGD groups. The resuscitation rates were similar between LR and AGD groups. There were no deaths during the experiments.

Table II shows rats' weight, surgery time, and blood removal during the experiment (mean  $\pm$  SEM). There were no differences among groups for weight, surgery time, and blood withdrawal.

Hemodynamic studies showed a small but measurable effect of AGD. Figure 1 shows that MAP in the sham control did not change during the experiment. MAP in the shock control, LR group, and AGD group fell to the same level during shock. Shock controls recovered somewhat after shock but continued to show much lower MAP than the others until the end of the experiment. MAP in both LR and AGD groups recovered to baseline within 10 minutes of beginning fluid resuscitation and exceeded baseline values during resuscitation. After resuscitation the MAP decreased gradually to a little lower than baseline. In the AGD group, MAP recovered more quickly than in the LR group during resuscitation, and the difference was statistically significant. Table III shows the differences of MAP between the LR and AGD groups in the first 8 minutes of resuscitation.

Expression of mRNA for TNF- $\alpha$ , IL-1 $\beta$ , and iNOS was most strongly exhibited in the shock group and the LR group. It was less strongly demonstrated in the AGD group. Figure 2 shows the number of animals exhibiting mRNA expression in the liver. Normal control rats were negative for TNF- $\alpha$  and IL-1 $\beta$  mRNA; only 1 rat was positive for iNOS mRNA. Only 2 or 3 of sham control animals ( $n = 9$ , each group) were positive for TNF- $\alpha$ , IL-1 $\beta$  or iNOS mRNA. In the shock controls

and in the LR group, most animals (7 or 8 in a total of 9) showed expression of mRNA for TNF- $\alpha$ , IL-1 $\beta$ , and iNOS. In the AGD group, only 3 rats were positive for TNF- $\alpha$ , IL-1 $\beta$ , and iNOS. The AGD group was not significantly different from the sham control group but was significantly different from the LR group for IL-1 $\beta$  and iNOS expression ( $p < .05$ ). All animals showed  $\beta$ -actin mRNA expression.

Figure 3 shows representative mRNA expression from each of the 5 experimental groups. A strong  $\beta$ -actin band indicated that adequate cDNA was obtained from all samples.

## DISCUSSION

When AGD was given during resuscitation from hemorrhagic shock, the MAP recovered more quickly than with LR alone. All animals received the same total allotted volume over the same time period. The mechanism of this circulatory response to AGD is not apparent from the present study. Studies by others have shown that AGD does not affect hemodynamics in normal sheep.<sup>7</sup> Investigators have found that intestinal blood flow is restored after hemorrhage with enteral glutamine administration, apparently by selective dilation of the premucosal arterioles, as in the suggestion by Flynn et al<sup>8</sup> that early enteral nutrition with glutamine after resuscitation from hemorrhagic shock may help to preserve intestinal mucosal integrity by restoring mucosal blood flow.

Our results showed that hemorrhagic shock and resuscitation with LR are associated with appearance of hepatic mRNA for TNF- $\alpha$ , IL-1 $\beta$ , and iNOS. These results indicate that proinflammatory gene activation

TABLE II  
Rats' weight, surgery time, and blood withdrawing

	Sham	Shock	LR	AGD
Weight, g	364 $\pm$ 5.6	358 $\pm$ 7.0	377 $\pm$ 15	356 $\pm$ 13
Surgery time, min	8.0 $\pm$ 0.2	7.2 $\pm$ 0.5	7.1 $\pm$ 0.4	6.4 $\pm$ 0.3
Blood withdrawing, mL	0	10.0 $\pm$ 0.7	11.0 $\pm$ 0.6	10.2 $\pm$ 0.9

AGD, alanine-glutamine dipeptide; LR, Ringer's lactate.

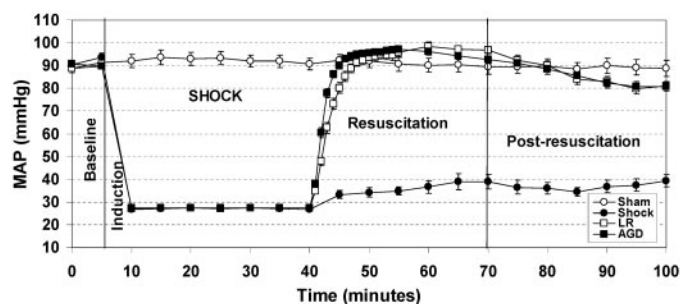


FIGURE 1. Time course of MAP during the experiment. Sham control group remained at baseline. Shock control, LR, and AGD groups fell to 25–30 mmHg for 30 minutes during the shock. LR and AGD groups recovered to baseline during resuscitation.



TABLE III  
MAP differences between Ringer's lactate (LR) and alanine-glutamine dipeptide (AGD) groups

Time, min	MAP (mm Hg)		<i>t</i> Test ( <i>p</i> value)
	LR	AGD	
40	27.2	27.4	.59
41	34.8	37.9	.014
42	47.8	60.4	<.01
43	62.6	77.8	<.01
44	73.2	86.1	<.01
45	80.2	89.9	.001
46	85.2	92.9	.004
47	88.8	94.2	.029
48	91.4	95	.129

MAP, mean arterial pressure.

occurs in the liver quite early after shock and resuscitation. Animals resuscitated with AGD showed a much less prominent mRNA response. The experimental hypothesis that AGD would suppress proinflammatory gene activation is supported by the results of the experiments.

Inflammatory cytokines have been implicated in tissue damage and mortality in hemorrhagic shock.<sup>9,10</sup> Resuscitation from hemorrhagic shock triggers an inflammatory response characterized by up-regulation of cytokine expression.<sup>11</sup> TNF- $\alpha$  and IL-1 $\beta$  have been reported to be derived predominantly from activated macrophages, and act *via* specific cell-membrane-bound receptors.<sup>12,13</sup> Work by Schroder et al<sup>14</sup> showed that AGD given in PN produced suppression of cytokine release in gut mononuclear cells and splenic macrophages compared with enteral-fed controls without AGD or glutamine.

In a review, Angele and Faist<sup>15</sup> indicated that increased plasma levels of proinflammatory cytokines act to depress macrophage function. *In vivo* administration of TNF- $\alpha$  and IL-1 $\beta$  induces a shocklike syndrome, similar to that observed after severe blood loss or sepsis. Angele and Faist<sup>15</sup> speculated that these cytokines may play a role in initiating the cascade of events that can lead to the development of multiple organ dysfunction after severe hemorrhagic shock.

iNOS is significantly up-regulated after hemorrhagic shock and postresuscitation in the liver. It is thought to be one of the major contributors to hepatic injury caused by severe hemorrhagic shock.<sup>2,16</sup> The mecha-

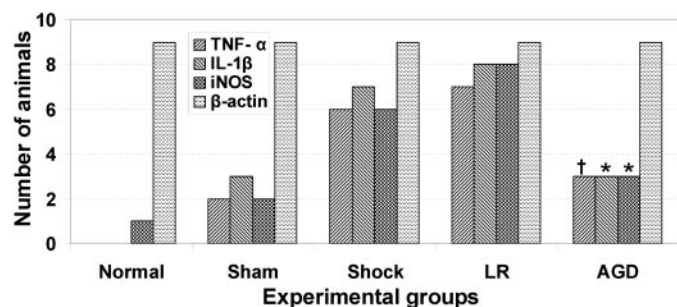


FIGURE 2. Number of animals expressing mRNA for TNF- $\alpha$ , IL-1 $\beta$ , iNOS and  $\beta$ -actin in different experimental groups. \**p* < .05; †*p* = .078 (AGD compared to LR group).

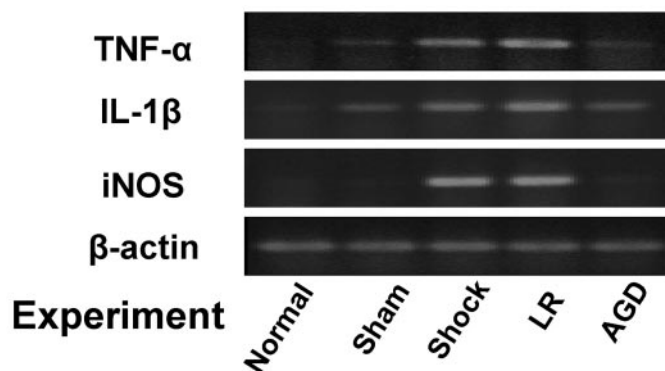


FIGURE 3. TNF- $\alpha$ , IL-1 $\beta$ , iNOS and  $\beta$ -actin mRNA expression. Taken from a representative rat in each group.

nism of up-regulation is unclear but could include hypoxia or the action of cytokines. Our results indicate that iNOS is not only up-regulated during shock but that it remains elevated after resuscitation.<sup>17</sup> Several studies have suggested that excessive NO production contributes to the organ injury associated with hemorrhagic shock and resuscitation.<sup>16,18–20</sup> The induction of iNOS is part of a complex inflammatory response, including activation of signaling proteins and cytokine expression, ultimately leading to morphologic and functional injury.<sup>17,21</sup>

Studies in postsurgical, critically ill patients have shown that parenteral supplementation of glutamine shortened hospital stay and improved nitrogen balance.<sup>3,22</sup> It has been suggested that glutamine protects against oxidant stress.<sup>23,24</sup> Other studies indicated that AGD modulates the immune response.<sup>25,26</sup> Depletion of glutamine correlates with altered phagocytic ability of monocytes *in vitro*. The expression of various antigens on human monocytes from apparently healthy volunteers is dependent on glutamine concentration in the cell culture medium.<sup>27</sup>

Resuscitation with AGD reduced liver mRNA expression for TNF- $\alpha$ , IL-1 $\beta$ , and iNOS compared with resuscitation with LR only (Figure 2). These results confirm our hypothesis that AGD suppresses postshock overexpression of mRNA for TNF- $\alpha$ , IL-1 $\beta$ , and iNOS.

## CONCLUSION

AGD has potential for treatment of hemorrhagic shock. The development of new pharmacologic strategies to minimize late tissue and organ damage has the potential to greatly improve the clinical treatment of hemorrhagic shock. Subsequent investigations will explore the mechanism of action of AGD and glutamine.

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# Effect of DHEA on the Hemodynamic Response to Resuscitation in a Porcine Model of Hemorrhagic Shock

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**Background:** Hemorrhagic shock is a major cause of death from trauma. Pharmacologic treatment has not been satisfactory. The objective of this study was to use a porcine model of hemorrhagic shock and resuscitation to access the hemodynamic effects of dehydroepiandrosterone (DHEA), an adrenal steroid hormone reported to improve cardiac function in patients.

**Methods:** Hemorrhagic shock was produced in 20- to 30-kg male Yorkshire pigs anesthetized with 2% isoflurane by withdrawing blood through a carotid cannula to a mean arterial pressure (MAP) of 40 to 45 mm Hg and maintaining that level for 60 minutes by further removals of

blood. Resuscitation was with 21 mL/kg Ringer's lactate (LR), with (n = 6) or without (n = 6) DHEA (4 mg/kg) dissolved in propylene glycol. The animals were killed after 7 days. Continuous cardiac output (CCO) was recorded using a modified Swan-Ganz catheter system. MAP, heart rate (HR), central venous pressure (CVP), and pulmonary arterial pressure (PAP) were measured every 5 minutes until 60 minutes postresuscitation. From MAP, CCO, HR, and CVP, we calculated total peripheral resistance (TPR), stroke volume (SV), and left ventricular stroke work (SW).

**Results:** The MAP, CCO, SV, and SW decreased significantly during hemor-

rhagic shock, and then gradually increased to baseline levels during and 1 hour after resuscitation. The TPR was increased during hemorrhagic shock, and then gradually decreased to baseline levels during and after resuscitation. DHEA administration was associated with no significant improvement.

**Conclusion:** DHEA when added to standard fluid resuscitation showed no added benefit as resumed by the hemodynamic response.

**Key Words:** Hemorrhagic shock, Dehydroepiandrosterone (DHEA), Continuous cardiac output.

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Dehydroepiandrosterone (DHEA) is the most abundant steroid hormone in plasma. Although it is an intermediary in the synthesis of testosterone and estrogen,<sup>1,2</sup> studies have indicated that administration of supplemental DHEA may be effective in the treatment of shock. Cardiac performance and organ blood flow are diminished in male rats after hemorrhage.<sup>3,4</sup> Androstenediol, one of the metabolites of DHEA, when given either subcutaneously or intravenously after hemorrhage has been reported to improve organ blood flow and function, and to prevent reperfusion injury.<sup>5-7</sup> Mice subjected to both trauma and hemorrhage received a subcutaneous injection of DHEA (4 mg/kg body weight), immediately before fluid resuscitation. This dose and route of DHEA administration have been previously found to be effective in restoring immune functional parameters in the

trauma-hemorrhage model.<sup>8</sup> Experiments in rats have showed that DHEA is associated with increased survival after hemorrhagic shock.

The objective of the present study was to assess the hemodynamic effects of DHEA in a porcine model of hemorrhagic shock and resuscitation. Because the cardiovascular responses of the pig are similar to those of humans, this study was thought to be an important preliminary to possible clinical application. This model has been extensively used in studies published from other investigators, and the porcine model has been used in our lab, as well. Although DHEA may be given either intravenously or subcutaneously, this study has used DHEA as part of the intravenously administered resuscitation regimen. The hypothesis of the study was that the use of DHEA during resuscitation would enhance the cardiovascular response, as measured by cardiac output, cardiac function, and peripheral resistance.

## MATERIALS AND METHODS

### Hemorrhagic Shock Model

Yorkshire pigs (male, 20 to 30 kg) were used in this study. All animals were kept in the animal care facility for several days before being used in the experimental study. The animals were anesthetized with xylazine (2 mg/kg) intramuscularly (IM) and ketamine (20 mg/kg) IM for induction, followed by endotracheal tube intubation and maintenance with isoflurane 1% to 3% and oxygen (3 L) during the remainder of the experiment.

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Vascular access was obtained in the following manner. An incision was made over the right side of the neck. Dissection was carried down to the carotid artery and jugular vein, and both were cannulated. The arterial cannula was connected to a blood pressure transducer, filled with lactated Ringer's solution containing 100 units of heparin per milliliter; blood pressure was monitored during the entire procedure. Through a catheter sheath placed in the jugular vein, a Swan-Ganz catheter modified for continuous cardiac output (CCO) measurement was placed, and connected to the monitoring device. This system allowed measurement of the cardiac output every minute throughout the experiment. Arterial blood pressure (ABP), mean blood pressure (MAP), central venous pressure (CVP), heart rate (HR), and pulmonary artery pressure (PAP) were also measured. Subsequently, calculations were made of left ventricular stroke volume ( $SV = (CO/HR) \times 1000 \text{ mL/beat}$ ), stroke work ( $SW = SV \times MAP \times 0.013 \text{ g}\cdot\text{m}$ ), and total peripheral resistance ( $TPR = ((MAP - CVP) / (CO \times 80)) \text{ dyne}\cdot\text{sec}/\text{cm}^5$ ). After cannulation, all cardiovascular measurements were performed for 15 minutes before shock was initiated.

Hemorrhagic shock was produced by withdrawing blood until the arterial pressure dropped below 45 mm Hg, maintaining the pressure at 40 to 45 mm Hg with further withdrawals of blood as required for a total of 60 minutes of shock.

Resuscitation solution was administered through the venous cannula. Ringer's lactate (LR) was administered (21 mL/kg), either with ( $n = 6$ ) or without ( $n = 6$ ) the addition of DHEA (4 mg/kg). After fluid administration, the shed blood (anticoagulated with citrate and kept warm) was returned to the animal. Resuscitation fluid and blood were given over a total of 25 to 30 minutes.

Monitoring was continued for 60 minutes after completion of resuscitation. The vessels were decannulated and the incision closed. The animal was allowed to awaken. Standard postoperative care, including the use of analgesics for discomfort, was performed. After 1 week, the animals were anesthetized, biopsies were taken for other studies (not reported), and the animals were killed using an overdose of barbiturates.

### Statistical Analysis

Data were analyzed using Excel. Student's  $t$  test was used to compare values for the hemodynamic measurements for the DHEA group and the control group, taken every 5 minutes through the protocol.

### Institutional Approval

All animal care and experimental procedures were performed according to the Guidelines of the Animal Care and Use Facility of the University of Missouri-Kansas City, and were in accordance with the guidelines of the American Association for the Accreditation of Laboratory Animal Care. The experimental protocols under which this work was per-

formed were approved by the Institutional Animal Care and Use Committee of the University of Missouri-Kansas City.

## RESULTS

All animals survived to one week. Results are presented in Table 1 and Figures 1 to 6. All animals showed a similar pattern. About half of the whole body blood volume was removed by the end of the shock period. The cardiac output, arterial pressure, stroke volume, and stroke work all decreased significantly during hemorrhagic shock and then gradually increased to baseline levels during resuscitation, continuing during the hour after resuscitation. Total peripheral resistance was elevated during hemorrhagic shock, and then gradually decreased to baseline levels during and after resuscitation.

Comparing the DHEA group with the control group, there were no significant differences ( $p > 0.05$ ).

## DISCUSSION

The cardiovascular effects of shock are as profound as the metabolic effects. Cardiac output is a useful method to evaluate cardiac function in clinical work, and experimental studies.<sup>4,6,9</sup> Cardiac output is useful in the large animal shock model, such as pig and dog.<sup>10-13</sup> This study applied the technique of continuous measurement of cardiac output using a thermodilution technique to shock and resuscitate. Our findings agree with those published by others in the porcine model, the canine model, and in patients.<sup>10-14</sup> The CCO was decreased during shock, rose to above baseline with resuscitation, then declined to baseline during the hour after resuscitation. Stroke volume and stroke work both reflected the changes in CCO. In this series of experiments, the animals did not show evidence of postresuscitation myocardial depression.

Continuous thermodilution cardiac output measurement has been considered the current bedside "gold standard" for cardiac output measurement.<sup>15</sup> However, it requires placement of a pulmonary artery catheter and questions have been raised regarding the risk-to-benefit ratio of this invasive technique.<sup>15-19</sup> Echocardiography can be used to provide noninvasive continuous measurement of cardiac output. It shows good agreement with continuous cardiac output measurement using thermodilution.<sup>16</sup> Kevin et al. concluded that echocardiography is a minimally invasive technique that has a good safety profile, is easy to use, and requires only minimal training. However, wide variations were observed with echocardiography in several studies, raising concerns about the precision of this technique. It appears unlikely that echocardiography will replace pulmonary artery catheter at present.<sup>15</sup> Other reviews concluded that the Swan-Ganz catheter monitoring has limitations but is an established technique for monitoring.<sup>17,18</sup> In the pig model, recording of echocardiography has been noted to be imprecise.<sup>19</sup> A major limitation of both techniques is that no continuous assessment of right ventricular parameters was possible. To overcome this problem, a new modified pulmonary artery catheter that continuously assesses right ventricular parameters has recently been introduced. The new continuous pulmonary artery cath-

**Table 1** DHEA and Ringer's Lactate (LR) Control for Pig Hemorrhagic Shock Model

	Mean Arterial Pressure (mm Hg)		Continuous Cardiac Output (L/min)		Total Peripheral Resistance (dyne-sec/cm <sup>5</sup> )		Stroke Volume (ml/beat)		Left Ventricular Stroke Work (gm)	
	LR	DHEA	LR	DHEA	LR	DHEA	LR	DHEA	LR	DHEA
Baseline	74.26 ± 5.33	73.30 ± 3.85	4.97 ± 0.83	3.60 ± 0.30	1082.10 ± 52.91	1393.35 ± 99.68	43.86 ± 4.38	34.73 ± 2.30	43.52 ± 7.12	31.17 ± 3.95
Postshock	71.75 ± 4.59	77.67 ± 4.77	5.92 ± 0.52	3.95 ± 0.35	898.67 ± 113.36	1439.48 ± 90.94	46.81 ± 5.84	31.23 ± 2.61	43.84 ± 6.56	31.98 ± 3.84
Rate (%)	96.62	105.96	119.11	109.72	83.05	103.31	106.73	89.92	100.74	102.60
postshock/ baseline)										
t test p value	0.52	0.47	0.33	0.43	0.34	0.82	0.77	0.2	0.81	0.68

eter system appears to be a valid and useful bedside monitoring device in the hemodynamic management of critically ill patients.<sup>17,20</sup>

Studies done by Chaudry and colleagues have shown that administration of DHEA (30 mg/kg) at the end of resuscitation prevented the deterioration of cardiac output that was seen in control animals.<sup>9</sup> Administration of DHEA also restored hepatocellular function and, in other experiments, prevented cytokine release.<sup>9,21</sup> It was suggested by the investigators that the improvement of cardiovascular function may be associated with attenuation in interleukin-6 levels and an increase in plasma nitrite/nitrate levels, and mediated via the estrogen receptors.<sup>5,6,9</sup>

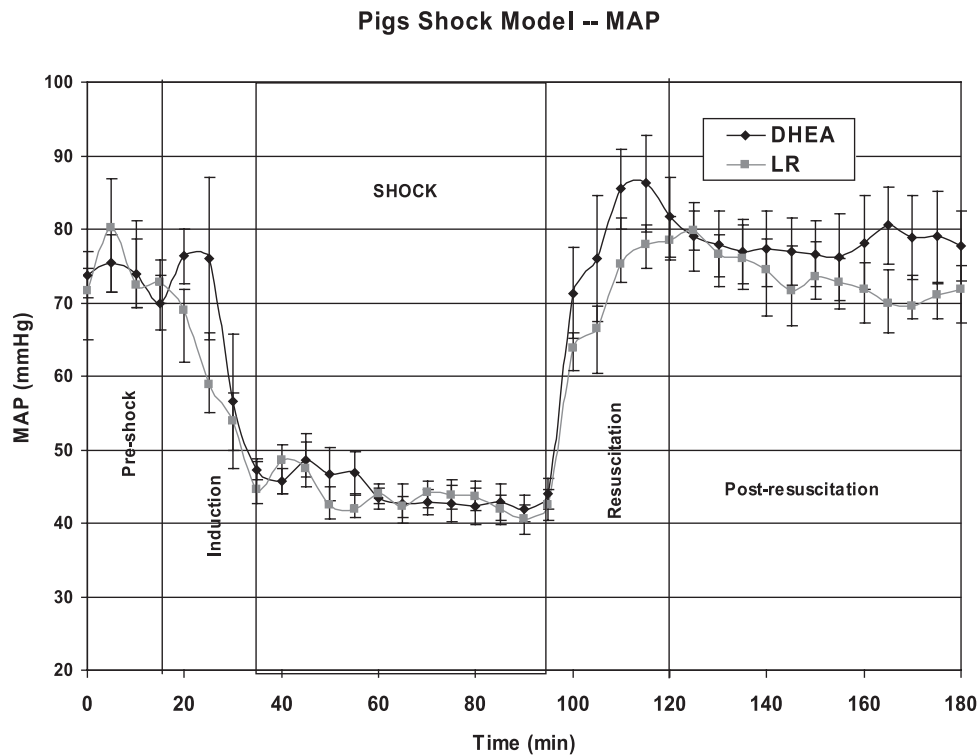
Studies in the mouse model showed that administration of DHEA prevented the depression of cell-mediated immune responses after a model combining trauma and hemorrhage. Moreover, studies demonstrated that DHEA treatment in male mice after trauma-hemorrhage improved immune response and decreased mortality from subsequent sepsis compared with control animals.<sup>1,8,22–26</sup> In a review, Jarrar et al. concluded that the use of DHEA has potential for possible use in patients, based on its use in the rodent models.<sup>21</sup>

Beishuizen et al. measured DHEA-sulfate (DHEAS) in 30 patients who were septic, 8 patients with multiple injuries, and 40 matched controls. They found that DHEAS was very depressed in septic shock, and somewhat less depressed in multiple injuries. Clinical studies suggested that DHEA might be a prognostic marker and a sign of exhausted adrenal reserve in critical illness.<sup>27</sup>

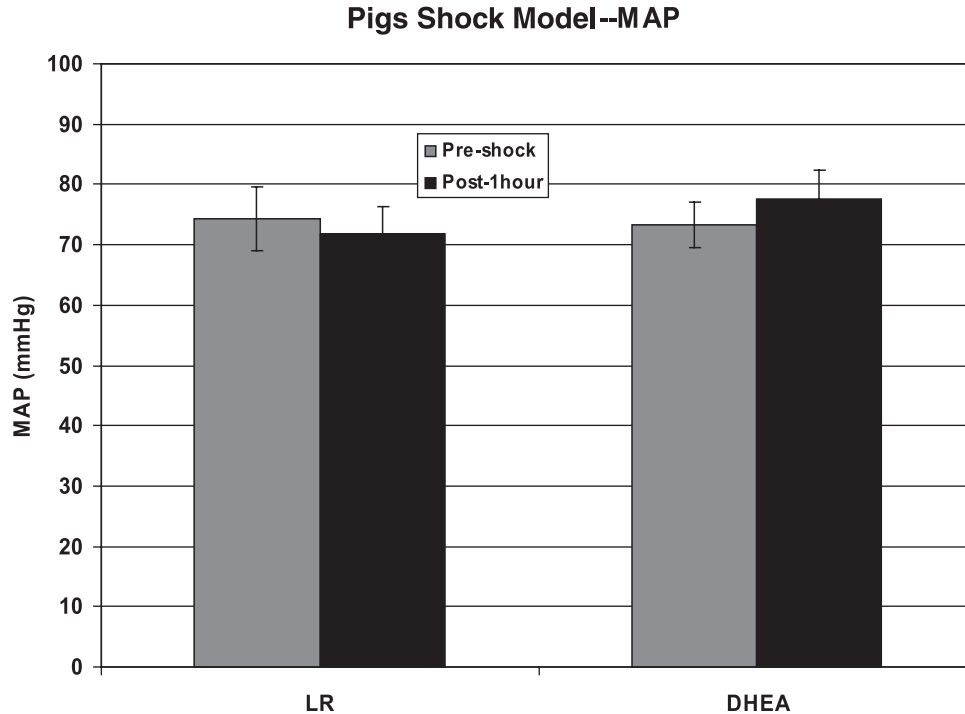
We have used a relatively modest dose of DHEA in this study. Considering the side effects of the drug given in larger doses which have been reported in human studies,<sup>28–31</sup> we thought that even if larger doses were effective, they would be of little use clinically. The dose we used is lower than used in the studies in the rat model, which showed that DHEA improved cardiac output.<sup>9</sup> It appears that there is species variance in the response to DHEA. In a pig model involving trauma and a delayed LPS challenge, animals were resuscitated with DHEA at larger doses (4 mg/kg, 10 mg/kg, and 20 mg/kg), and dosed again at 24, 48, and 72 hours. DHEA administration failed to blunt the associated systemic inflammatory response and pulmonary failure.<sup>32</sup> The results of the present study then are consistent with other findings in the pig model.

Hemorrhagic shock is an important clinical problem and cannot be presently treated effectively with drugs. The development of new pharmacologic strategies to minimize late tissue and organ damage will greatly improve the clinical treatment of hemorrhagic shock. This study was done as part of a series of investigations designed to define optimal resuscitation fluids and potentially useful supplements for treatment of hemorrhagic shock. The object of improving resuscitation is to reduce postresuscitation cellular damage, consequent organ dysfunction, and mortality. Because the available experimental evidence suggests that currently promising interventions may work through different mech-





**Fig. 1.** Both Ringer's lactate and DHEA treatment mean arterial pressure decreased significantly during hemorrhagic shock, and then gradually increased to baseline levels during and 1 hour after resuscitation.

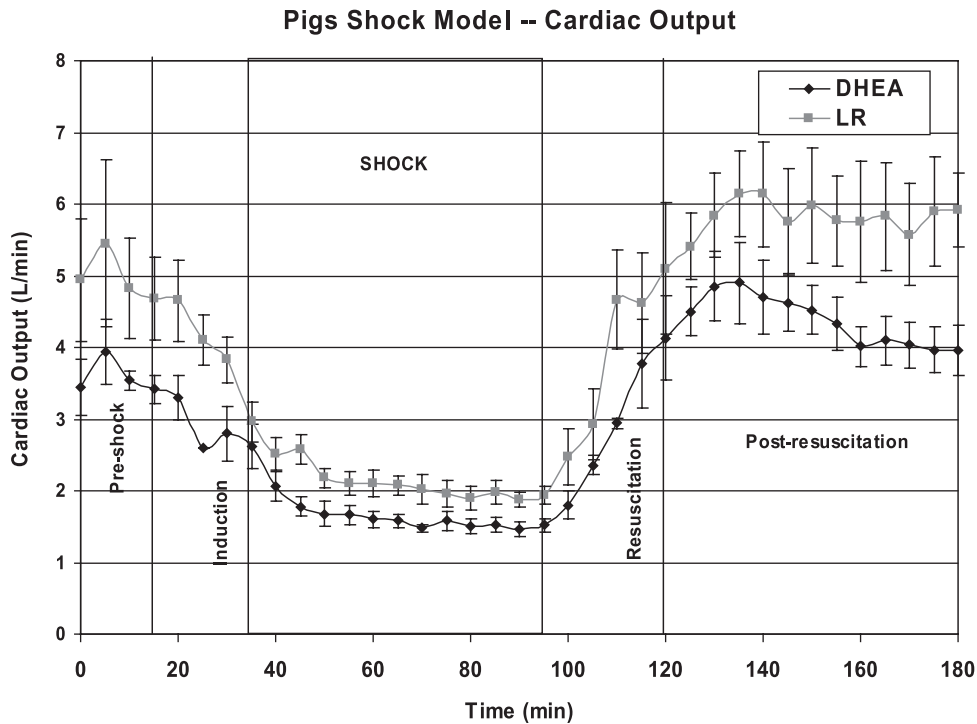


**Fig. 2.** Ringer's lactate and DHEA treatment mean arterial pressure (MAP) at preshock and 1 hour postresuscitation. The results indicate that preshock and 1 hour postresuscitation have no significant difference between groups.

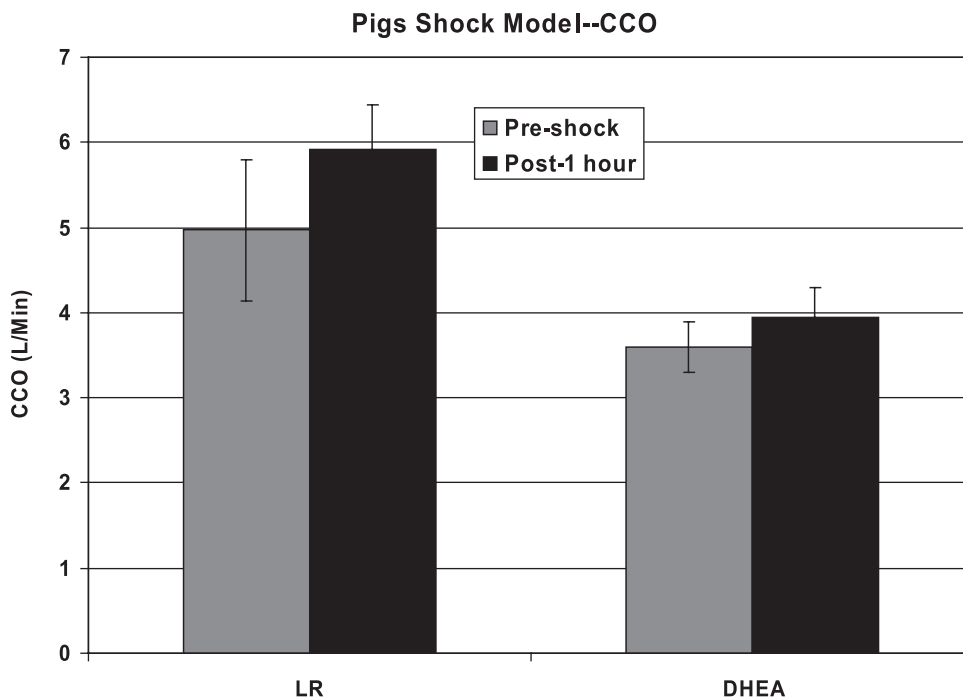
anisms, there is a critical need to determine the extent to which combinations of these strategies might be employed to further improve outcomes.

## CONCLUSIONS

The hypothesis of this study was that DHEA, when added to standard fluid resuscitation, would enhance the



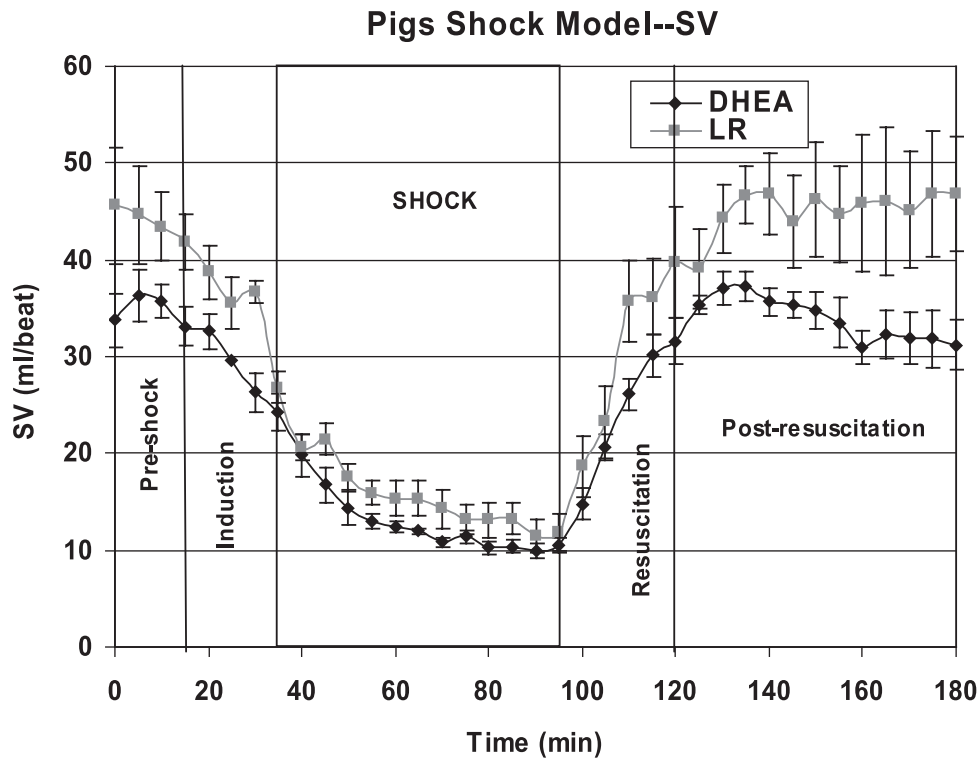
**Fig. 3.** Both Ringer's lactate and DHEA treatment continuous cardiac output decreased significantly during hemorrhagic shock, and then gradually increased to baseline levels during and 1 hour after resuscitation.



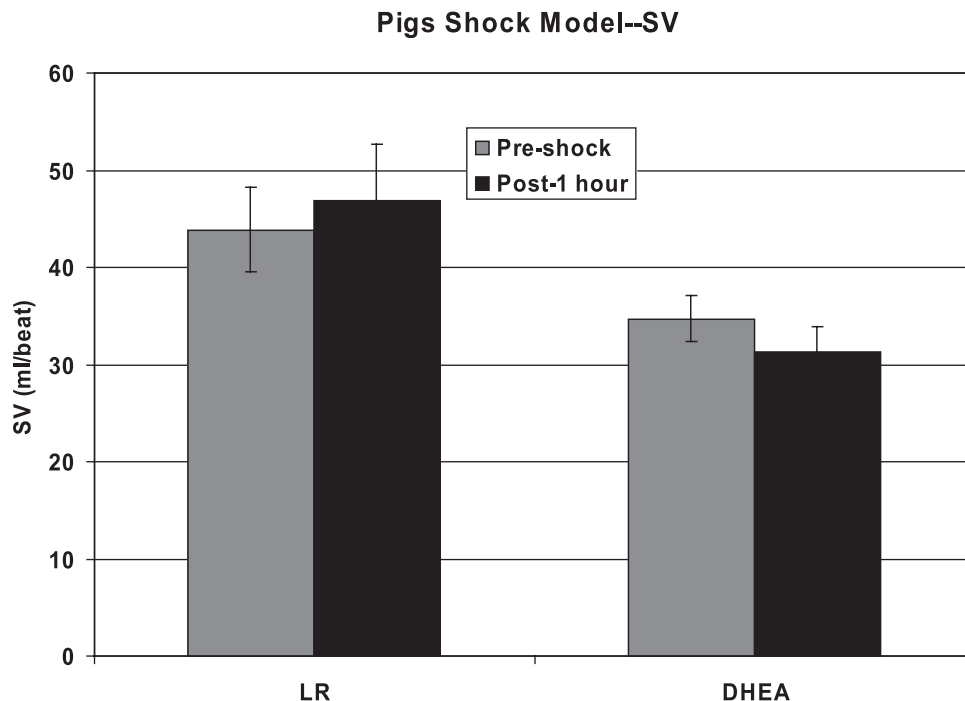
**Fig. 4.** Ringer's lactate and DHEA treatment continuous cardiac output (CCO) at preshock and 1 hour postresuscitation. The results indicate that preshock and 1 hour postresuscitation CCO have no significant difference between groups.

cardiovascular response. However, the evidence showed clearly that DHEA has no effect upon the early cardiovascular response to resuscitation after hemorrhagic shock. Clinical studies cannot be recommended on the basis of these find-

ings. However, this investigation used only a single, relatively modest dose of DHEA (4 mg/kg), and does not rule out the possibility that higher doses might have produced a hemodynamic response. It should be noted that this study



**Fig. 5.** Both Ringer's lactate and DHEA treatment stroke volume decreased significantly during hemorrhagic shock, and then gradually increased to baseline levels during and 1 hour after resuscitation.



**Fig. 6.** Ringer's lactate and DHEA treatment stroke volume at preshock and 1 hour postresuscitation. The results indicate that preshock and 1 hour postresuscitation stroke volumes have no significant difference between groups.

did not include the measurement of inflammatory or metabolic responses to shock and resuscitation, and hence cannot exclude the possibility that DHEA is beneficial in

other ways, as previous investigations have suggested. The present study employed continuous measurement of cardiac output in the porcine shock model. This methodology

was found to be useful in the study of hemorrhagic shock and resuscitation.

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## Hemorrhagic Shock in the Rat: Comparison of Carotid and Femoral Cannulation

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## **Abstract**

*Background.* The reservoir model of rat hemorrhagic shock is widely used. In this model, either the carotid or femoral artery can be cannulated to withdraw blood and measure pressure. In animals undergoing hemorrhage using the carotid approach, we observed seizure activity during the post-shock period, suggesting some degree of brain damage. The hypothesis of the present study is that survival in a model of severe hemorrhagic shock would be higher with femoral cannulation than with carotid cannulation.

*Materials and methods.* All animals (n=90) were anesthetized with isoflurane using an anesthesia vaporizer while breathing spontaneously. In group 1, the left carotid artery and jugular vein were cannulated; in group 2, the left femoral artery and vein were cannulated. Following a period of hemorrhagic shock (20-30mmHg for 30, 60, or 90 minutes), resuscitation was performed through the venous cannula by giving L-Lactated Ringer's (21ml/kg) and returning the shed blood.

*Results.* In the carotid cannulation group, nearly 50% of the animals had seizures after resuscitation, and most of those animals died following the seizures. The 24-hour survival rate in the femoral artery cannulation group was significantly higher than in the carotid artery cannulation group. Femoral cannulated animals had no seizures following reperfusion.

*Conclusions.* Femoral artery cannulation was associated with considerably better survival than carotid artery cannulation in this rodent model of hemorrhagic shock. The

occurrence of seizures in animals undergoing carotid cannulation suggests brain damage from inadequate cerebral perfusion or subsequent reperfusion damage.



## INTRODUCTION

The reservoir model of hemorrhagic shock using the rat is widely used in shock research. Cannulation techniques have used either the carotid or femoral artery. Carotid cannulation in the normal rat appears to produce no defects in cerebral perfusion, and the artery can be ligated at the conclusion of the procedure without apparent ill effects. However, this is not necessarily true during severe hemorrhagic shock. In a number of animals undergoing severe hemorrhage, we have observed seizure activity during the post-shock period, suggesting some degree of brain damage.

The hypothesis of the present study is survival in a rat model of severe hemorrhagic shock is higher with femoral cannulation than with carotid cannulation.

## MATERIALS AND METHODS

Male Sprague-Dawley rats (n=90, 350±30g) were randomly assigned to two groups. In group 1(n=70), the right carotid artery and jugular vein were cannulated. In group 2 (n=20), the left femoral artery and vein were used. [All animals were anesthetized with isoflurane](#) using an anesthesia vaporizer while breathing spontaneously. Following a period of hemorrhagic shock (20-25 or 25-30mmHg for 30, 60, or 50-90 minutes), resuscitation was done through the venous cannula by giving L-lactated Ringer's solution (21ml/kg) and returning the shed blood. Animals were monitored closely, and the survival rate was recorded at the end of 24, 48, and 72 hours.

All animal care and experimental procedures were carried out according to the Guidelines of the Animal Care and Use Facility of the University of Missouri-Kansas City.

## RESULTS

As shown in Table 1, rats (n=27) were cannulated using the carotid arteries and were subjected to hemorrhagic shock, mean arterial pressure (MAP) = 20-25mmHg, for 30 minutes. The survival results showed that seizures were more common in animals surviving <24 hours than in those surviving >24 hours ( $p<0.05$ ). That is, seizures were associated with earlier death following shock.

In comparing carotid (n=39) and femoral (n=15) cannulation, rats were subjected to MAP of 20-30mmHg for 60 minutes. The 24 hour survival rate (Table 2) in femoral cannulated animals (15/15) was significantly higher than in carotid cannulated animals (16/39). The carotid cannulation group had a significant occurrence of seizures (18/39), while the femoral cannulation group had none (0/15). Again, seizure rate was significantly higher in the group of animals that died within 24 hours (Table 3,  $p<0.05$ ).

Table 4 shows the comparison of carotid (n=4) and femoral (n=5) cannulation in animals subjected to MAP of 20-25mmHg for 50-90 minutes. All of the four rats in the carotid artery cannulation group had seizures and died within 24 hours. In contrast, all of the five rats belonging to the femoral artery cannulation group survived longer than 24 hours without seizure activity, as shown in.

## DISCUSSION

These survival studies have shown an advantage to femoral artery cannulation in the rat model of hemorrhagic shock. Investigators have noticed that overweight and older rats have a markedly higher mortality rate following hemorrhagic shock . The

present results show rats cannulated through the carotid artery in a hemorrhagic shock model also have increased mortality compared with rats cannulated through the femoral artery.

Cannulation technique is within the control of the investigator, and appears to represent a confounding factor in the rodent reservoir shock model. In studies by others, using carotid cannulation, MAP was maintained at 20-25mmHg during hemorrhagic shock in the rat model. There was a high mortality following a 30 minute shock period [1]. Previous studies from our laboratory showed that with femoral artery cannulation and shock at MAP=20-25mmHg for 30 minutes, survival was nearly 100% even in animals receiving no resuscitation (shocked, non-resuscitated, controls; unpublished data). Lethal rat hemorrhagic models from different investigators have shown that MAP was lower and the shock time was longer using femoral cannulation than with carotid cannulation [2-5]. In one study, no functional or histologic brain damage was noted in rats cannulated through the femoral artery and bled to a MAP of 40 mmHg for 60 minutes, or a MAP of 30 mmHg for 45 minutes [5].

## **CONCLUSION**

Femoral artery cannulation was associated with considerably better survival than carotid artery cannulation in this model of severe hemorrhagic shock. The occurrence of seizures in animals undergoing carotid cannulation suggests cerebral damage from inadequate cerebral perfusion combined with reperfusion.

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TABLE 1

Cannulated Carotid Artery and Jugular Vein

Survival Time	>72 hours	24-48 hours	<24 hours
Weight (gram)	359±13	363±17	359±16
Bleeding (ml)	11.7±0.7	12.1±0.5	12.9±0.4
Seizure animals	1/7	2/7	10/13*

Shock at 20-25mmHg & 30minutes. Data expressed as mean±SEM.

\*P<0.05 (survival <24 hours vs survival >24 hours).

TABLE 2

Comparison of Carotid and Femoral Cannulation

Cannulated artery	Carotid A	Femoral A
Survival at 24 hours	16/39	15/15*
Weight (gram)	355±6	359±12
Cannulation time (min)	7.2±0.2	9.6±0.6
MAP (mmHg)	25-30	20-30
Blood removed (ml)	13.6±0.2	14.3±0.6
Seizures	18/39	0/15*

Shock at 25-30mmHg pressure, 60 minutes. Data expressed as mean±SEM.

\*p<0.05 (femoral cannulation vs carotid cannulation)

TABLE 3

Cannulated Carotid Artery and Jugular Vein

Survival Time	>24 hours	<24 hours
Weight (gram)	360±9	352±7
Caunulated time (min)	7.3±0.2	7.1±0.4
Bleeding (ml)	13.8±0.4	13.4±0.3
Seizure animals	4/16	14/23*

Shock at 25-30mmHg pressure, 60 minutes. Data expressed as mean±SEM.

\*P< 0.05 (survival < 24 hours vs survival >24 hours)



TABLE 4

Comparison of Carotid and Femoral Cannulation

Cannulated artery	Carotid A	Femoral A
24 hours Survival	0/4	5/5*
Weight (gram)	353±14	346±10
Blood removed (ml)	12.8±1.5	13.6±1.4
Seizures	4/4	0/5*

Shock at MAP=20-25 mmHg, 50-90 minutes. \*p<0.05 (femoral cannulation vs carotid cannulation)